REMARKS

Claims 1-25 were pending in the application. Claims 19-25 have been canceled without prejudice, claims 1-3, 6-10, 15, 17, and 18 have been amended, and new claim 26 has been added. Accordingly, upon entry of the amendments presented herein, claims 1-18 and 26 will remain pending in the application.

Support for the amendments to the claims may be found throughout the specification and claims as originally filed. Specifically, support for the amendments to claim 1 may be found at, for example, page 10, lines 19-30 of the specification; support for the amendments to claim 6 may be found at, for example, page 10, lines 19-30 of the specification; and support for new claim 26 may be found in, for example, originally filed claim 6 and claim 18. *No new matter has been added by the foregoing claim amendments or the addition of new claim 26.*

Any amendments to and/or cancellation of the claims are not to be construed as an acquiescence to any of the rejections set forth in the instant Office Action, and were done solely to expedite prosecution of the application. Applicants hereby reserve the right to pursue the subject matter of the claims as originally filed in this or a separate application(s).

Specification

The Examiner has objected to the specification as "failing to provide proper antecedent basis for the claimed subject matter." In particular, the Examiner is of the opinion that "[t]he structure of instant claim 18 is omitted from the specification whereas the chain of the compound of the instant claim 18 is different than the compound of formula II (spec., p12)."

Applicants respectfully submit that the amendments to the specification presented herein render the Examiner's objection moot and, accordingly, respectfully request reconsideration and withdrawal of the foregoing objection.

Claim Objections

The Examiner has objected to claim 17 "as being of improper dependent form for failing to further limit the subject matter of the previous claim."

Applicants respectfully submit that the amendment to claim 17 to specify that L is a linker group and X is a biotinyl moiety has rendered the foregoing rejection moot. Accordingly, Applicants respectfully request reconsideration and withdrawal of the objection to claim 17.

The Examiner has objected to claim 19 because "the steps of claim 19 are (a) and (2) and should either be altered to read (a) and (b) or (1) and (2)."

Applicants respectfully submit that the cancellation of claim 19 has rendered the foregoing rejection moot. Accordingly, Applicants respectfully request reconsideration and withdrawal of the objection to claim 19.

The Examiner has also objected to claims 17 and 18 because "instant claim 17 and 18 fail to end in a period as required."

Applicants respectfully submit that the amendments to claims 17 and 18 to include a period at the end of each claim has rendered the foregoing rejection moot. Accordingly, Applicants respectfully request reconsideration and withdrawal of this objection to claims 17 and 18.

Rejection of Claims 1-3, 9, 10, 19, 20, and 22 Under 35 U.S.C. § 112, Second Paragraph

The Examiner has rejected claims 1-3, 9, 10, 19, 20, and 22 under 35 U.S.C. § 112, second paragraph, as allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." In particular, the Examiner is of the opinion that

> [i]t is unclear as to which test compound or biological target to use for the method of measuring the ability of a test compound to inactivate a biological target in the instant claims 1-3, 9, 10, 19, 20, and 22. The administration of different compound will vary with regards to dose or the biological target of interest.

Applicants respectfully traverse this rejection on the grounds that the claims are clear and definite. However, in the interest of expediting prosecution and allowance of the pending

claims, and in no way acquiescing to the validity of the Examiner's rejection, the claims have been amended to specify that the test compound is an inhibitor of a biological target. Accordingly, the foregoing rejection has been rendered moot and Applicants respectfully request that this rejection of claims 1-3, 9, 10, 19, 20, and 22 under 35 U.S.C. §112, second paragraph, be reconsidered and withdrawn.

Rejection of Claims 19-25 Under 35 U.S.C. §102

The Examiner has rejected claims 19-25 under 35 U.S.C. § 102(b) as allegedly being anticipated by Turk *et al.* ((1999) *Chem. Biol.* 6:823-833). In particular, the Examiner is of the opinion that Turk *et al.* teaches the

> treatment of bovine aortic endothelial cells with fumagillin analog, TNP-470 that are then lysed for determination of unbound MetAP2. These lysates were treated with biotin-fumagillin, labeling MetAP2 protein that remained unbound following TNP-470 treatment. Bound biotin was detercted by probing the membrane with streptavidin-horseradish peroxidase, and the signal was competed by cell treatment with concentrations of TNP-470 (p824, results). The inhibition of MetAP2 examined in several human cell lines, such as HeLa, Jurkat T lymphocytes and HT1081C (p825, paragraph 1).

Without acquiescing to the validity of the Examiner's rejection and solely in the interest of expediting prosecution and allowance of the pending claims, Applicants have canceled claims 19-25, thereby rendering the foregoing rejection moot. Accordingly, Applicants respectfully request that this rejection of claims 19-25 under 35 U.S.C. §102(b) be reconsidered and withdrawn.

Rejection of Claims 1-17 and 19-25 Under 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-17 and 19-25 under 35 U.S.C. §103(a) as being unpatentable over Turk et al. ((1999) *Chem. Biol.* 6:823-833) and Amitai *et al.* (WO 02/065977). In particular, the Examiner is of the opinion that Turk *et al.* teaches the

> treatment of bovine aortic endothelial cells with fumagillin analog, TNP-470 that are then lysed for determination of unbound METAP2. These

> lysates were treated with biotin-fumagillin, labeling METAP2 protein that remained unbound following TNP-470 treatment. Bound biotin was deterected by probing the membrane with streptavidin-horseradish peroxidase, and the signal was competed by cell treatment with concentrations of TNP-470 (p824, results). The inhibition of METAP2 examined in several human cell lines, such as HeLa, Jurkat T lymphocytes and HT1081C (p825, paragraph 1).

The Examiner is also of the opinion that Amitai *et al.* teach

> the treatment of recombinant human acetylcholinesterase, fetal bovine-serum-AchE or purified human plasma butyrylcholinesterase with test compound and their activity measured. The in vivo inhibition of whole blood Che in mice was measured by injecting the tested compounds to the animals then sampling the blood via eye orbit vein (p76).

Based on these characterizations of the cited art, the Examiner concludes that

> [a]t the time of the invention it would have been obvious to one ordinarily skilled in the art to use the method of measuring the inhibition of MetAP2, such as disclosed by Turk *et al.* (*Chem. Biol.* **1999**, 6, 823-833) by administering the fumagillin analog to mice then collecting the whole blood, as disclosed by Amitai *et al.* (WO 02/065977) since mice have a genetic similarity to humans and are frequently utilized for in vivo testing and in vivo testing is the next logical application after studying the properties of a compound in vitro.

With respect to claims 19-25, cancellation of these claims has rendered the Examiner's rejection moot. With respect to claims 1 and 6 and claims dependent therefrom, and new claim 26, Applicants respectfully traverse the Examiner's assertion that the proposed combination of the above-cited references would have rendered the claimed invention obvious to the ordinarily skilled artisan at the time of the invention. Reconsideration and withdrawal of the rejection in light of the following discussion is respectfully requested.

Claim 1, and claims dependent therefrom, are directed to methods of measuring the ability of a test compound which is an inhibitor of a biological target to inactivate the biological target in a cell of a subject, comprising the steps of (a) ***administering the test compound to a subject***, such that any of the biological target in the subject's body which reacts with the test compound is inactivated and any of the biological target which does not react with the test

compound is free, (b) ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject,*** (c) determining the amount of the free biological target within each of the plurality of biological samples, or fractions thereof, and (d) comparing the amounts determined in step (c) with the amount of free biological target in a control sample, wherein a decrease in the amount of free biological target each of the biological samples determined in step (c) compared to the amount determined in the control sample provides a measure of the amount of inactivated biological target in each of the biological samples, or fractions thereof. Claim 6, and claims dependent therefrom, are directed to methods for determining the extent of inactivation of MetAP-2 in a biological sample, or fraction thereof, derived from a subject, comprising the steps of (a) ***administering a test compound which is an inhibitor of MetAP-2 to the subject,*** wherein any MetAP-2 in the body of the subject that reacts with the test compound is inactivated MetAP-2 and any MetAP-2 that does not react with the test compound is free MetAP-2, (b) ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject,*** and (c) determining the amount of free MetAP-2 within each of plurality of the biological samples, or fractions thereof, and (d) comparing the amounts determined in step (c) with the amount determined in a control sample, wherein a decrease in the amounts in each of the samples determined in step (c) compared to the amount determined in step (d) is a measure of the extent of inactivation of MetAP-2 in each of the biological samples, or fractions thereof. New claim 26 further specifies the structure of the MetAP-2 inhibitor.

To establish a *prima facie* case of obviousness, it is necessary for the Examiner to present evidence, preferably in the form of some teaching, suggestion, incentive or inference in the applied references, or in the form of generally available knowledge, that one having ordinary skill in the art would have been motivated to make the claimed invention and would have had a reasonable expectation of success in making the claimed invention. Under section 103, "[b]oth the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure" (*Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.* 927 F.2d 1200, 1207, 18 USPQ2d 1016 (Fed. Cir. 1991), quoting *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed Cir. 1988)). Moreover, when a combination of references are used to establish a *prima facie* case of obviousness, the Examiner must present evidence that one having ordinary

skill in the art would have been motivated to combine the teachings in the applied references in the proposed manner to arrive at the claimed invention. See, e.g., *Carella v. Starlight Archery*, 804 F.2d 135, 231 USPQ 644 (Fed. Cir. 1986); and *Ashland Oil, Inc. v. Delta Resins and Refractories, Inc.*, 776 F.2d 281, 227 USPQ 657 (Fed. Cir. 1985). Finally, the prior art reference (or references when combined) must teach or suggest all of the claim limitations (M.P.E.P. 2143).

Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness, since the cited references, alone or in combination, fail to teach or suggest all of the claim limitations and furthermore fail to provide the necessary motivation for the ordinarily skilled artisan to measure the ability of a test compound to inactivate the biological target in a plurality of biological samples from a subject (claim 1) or to determine the extent of inactivation of MetAP-2 in a plurality of biological samples derived from a subject, (claims 6) by ***administering the test compound to a subject***, such that any of the biological target in the subject's body which reacts with the test compound is inactivated and any of the biological target which does not react with the test compound is free, ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject***, or determining the amount of free MetAp2 using ***a quantifiable irreversible MetAP-2 inhibitor recited in*** claim 26.

In particular Applicants submit that Turk *et al.* teach a method for determining the ability of a test compound to inactivate MetAP2 in ***<u>cultured</u> bovine aortic endothelial cells*** (BAECs) (page 824, right-hand column, second paragraph). As evidenced by the Examiner's own admissions, there is no teaching or suggestion in Turk *et al.* to ***administer a test compound to a subject***, or removing a biological sample from the subject, as required the pending claims. Moreover, Turk *et al.* fail to teach or suggest ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject*** as required by Applicants' claims. Thus, Turk *et al.* fail to teach or suggest the claimed methods.

Moreover, the secondary reference relied on by the Examiner, Amitai *et al.*, does not make up for the deficiencies in the primary reference. Specifically, Amitai *et al.* is directed to bifunctional chimeric compounds for the treatment of CNS disorders. In one of their examples, Amitai *et al.* merely disclose testing their compounds for the ability to inhibit blood

cholinesterase activity, namely, Amitai *et al.* test for cholinesterase activity in a single sample. However, Amitai *et al.* fail to teach or suggest that the inhibition of biological target by a test compound can be assayed in ***a plurality of biological samples from a subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject***. Amitai *et al.* also fail to teach or suggest use of a quantifiable irreversible MetAP2 inhibitor of the structure recited in claim 26. Thus, Amitai *et al.*, also fail to teach or suggest the claimed methods.

In view of the foregoing, it is evident that, Turk *et al.* and/or Amitai *et al.*, either alone or in combination, fail to teach or suggest the claimed invention and, thus, fail to render the claimed invention obvious.

Regarding the motivation to combine these references, the Examiner has failed to present evidence in the form of some teaching, suggestion, incentive or inference in the applied references that would have motivated one of ordinary skill in the art to use the *in vitro* assay of Turk *et al. in vivo*. Thus, the Examiner has also failed to provide evidence of a motivation to combine the teachings of Turk *et al.* and Amitai *et al.*, as relied upon for the §103 rejection.

The Examiner has failed to point to any teaching in the cited references which would impel one of ordinary skill in the art to make the claimed invention. The mere fact that references can be combined or modified does not render the resultant combination obvious, unless the prior art also suggests the desirability of the combination. M.P.E.P 2143.01. The prior art must suggest "to those of ordinary skill in the art that they *should* make the claimed composition or device, or carry out the claimed process" and "[b]oth the suggestion and the reasonable expectation of success ***must be founded in the prior art, not in the applicant's disclosure*** (emphasis added)." *In re Dow Chemical Co.* 837 F.2d 469. 473, 5 U.S.P.Q.2d 1529, 1531 (Fed.Cir. 1988).

Thus, the mere fact that Turk *et al. could* have been combined with the teachings of Amitai *et al.* does not mean that the combination of the teachings would have been obvious to one of ordinary skill in the art at the time of Applicants' invention. In fact, Applicants submit that ***one of ordinary skill in the art would not have been motivated to look beyond the teachings of Turk et al. for methods to measure the ability of a test compound to inactivate a biological target or methods to determine the extent of inactivation of MetAP2 since Turk et***

al. had already achieved this goal. Thus, it is evident that the Examiner's proposed combination of references is improper, as it is based on impermissible hindsight reconstruction.

It is Applicants position that the teachings of the cited references relied upon by the Examiner to combine the references are legally insufficient to provide the requisite motivation. With regard to the necessary legal standard, Applicants refer the Examiner to *In re Vaeck* (*In re Vaeck* 947 F.2d 488. (Fed. Cir. 1991)) where the CAFC upheld the non-obviousness of a biotechnology invention. In Vaeck the invention was drawn to "a chimeric (*i.e.*, hybrid) gene comprising (1) a gene derived from a bacterium of the *Bacillus* genus whose product is an insecticidal protein, united with (2) a DNA promoter effective for expressing the Bacillus gene in a host cyanobacterium, so as to produce the desired insecticidal protein (footnote omitted)." *Id* at page 490. The prior art was applied in various combinations against the claims. The primary reference (Dzelzkalns) taught the expression of a chimeric gene comprising a chloroplast promoter sequence fused to a gene encoding the enzyme chloramphenicol acetyl transferase (CAT) in cyanobacteria. The secondary references taught, *inter alia*, "expression of genes encoding certain *Bacillus* insecticidal proteins" in other host cells; "the initiation specificities in vitro of DNA-dependent RNA polymerases purified from two different species of cyanobacteria (footnote omitted);" and "a host-vector systems for gene cloning in the cyanobacterium." *Id* at page 491. The Examiner's position was that:

> it would have been obvious to one of ordinary skill in the art to substitute the Bacillus genes [which had been expressed in heterologous hosts in the teachings of the prior art] for the CAT gene in the vectors of Dzelzkalns in order to obtain high level expression of the *Bacillus* genes in the transformed cyanobacteria. The Examiner further contended that it would have been obvious to use cyanobacteria as heterologous hosts for expression of the claimed genes due to the ability of cyanobacteria to serve as transformed hosts for the expression of heterologous genes.

Id at page 492. The CAFC disagreed with the Examiner's position and found that the teachings of the prior art cited in Vaeck were not sufficient to support the interchangeability of bacteria and cyanobacteria as host organisms for the expression of heterologous insecticidal proteins. The court stated that "there is no suggestion in Dzelzkalns, the primary reference cited against all claims, of substituting in the disclosed plasmid a structural gene encoding *Bacillus* insecticidal proteins for the CAT gene utilized for selection purposes. The expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious the

expression of unrelated genes in cyanobacteria." *Id* at page 493. The court further stated that while the prior art disclosed "expression of *Bacillus* genes encoding insecticidal proteins in certain transformed bacterial hosts, nowhere do these references disclose or suggest expression of such genes in transformed cyanobacterial hosts . . . [w]hile it is true that bacteria and cyanobacteria are now both classified as procaryotes, that fact alone is not sufficient to motivate the art worker as the PTO contends. *Id* at pages 493 and 494.

Similar to the situation in *In re Vaeck*, it is Applicants' position that despite the fact that the prior art contained separate elements of the invention (*i.e.*, Turk *et al.* who make a determination of unbound MetAP2 *in vitro* and, and Amitai who determine the activity of cholinesterase from a sample treated *in vivo* with a cholinesterase inhibitor), these individual teachings are insufficient to establish the obviousness of the claimed invention absent some teaching or suggestion in the art to combine and modify the teachings of those references to arrive at the claimed invention. It is Applicants' position that the motivation relied upon by the Examiner is legally insufficient to establish the requisite suggestion to combine the references.

Moreover, Applicants submit that in contrast to the Examiner's assertion that transitioning an assay method from an *in vitro* assay format to an *in vivo* assay format is not as straightforward and simple as the Examiner presumes it to be. In support of this position, Applicants point the Examiner to Appendices A-C, submitted herewith. In particular, Appendix A (Smolinski and Petska (2003) *Food Chem. Toxicol.* 41:1381) teach that "cell culture data can only be used to approximate potential effects in animals" (see, *e.g.*, Abstract). Similarly, Appendix B (Stewart and Vandevoort (2007) *J. Clin. Endocrinol. Metab.* 82:3078) teaches that although the particular cell culture system utilized in the study "mimics the profile of ovarian steroids and relaxin seen in serum during the nonconceptive luteal phase", "the relative magnitude of hormones was not the same as seen *in vivo*" (see, *e.g.*, Abstract). Appendix C (Tiffany-Castiglioni, *et al.* (1999) *Toxicol. Sci.* 51:178) also cautions of the extrapolation of data from *in vitro* systems to *in vivo* systems (see entire document, especially, page 179, right-hand column, first full paragraph and page 180, right-hand column, first and second full paragraphs).

Accordingly, based on the *in vitro* method of Turk *et al.* one of skill in the art would not have been motivated to make the claimed invention nor would have had a reasonable expectation of success in making the claimed invention.

In summary, Applicants respectfully submit that, contrary to the Examiner's assertions, the ordinarily skilled artisan at the time of Applicants' invention would not have been motivated nor have reasonably expected to succeed in arriving at Applicants' invention. For the foregoing reasons, rejection of the claimed invention is believed to be improper and Applicants respectfully request that it be withdrawn.

Rejection of Claims 1-17 and 19-25 Under 35 U.S.C. § 103(a)

The Examiner has also rejected claims 1-17 and 19-25 under 35 U.S.C. §103(a) as being unpatentable over Griffiths, *et al.* ((1998) *Proc. Natl. Acad. Sci., USA* 95:15183-15188) and Morain *et al.* ((2000) *Br. J. Clin. Pharmacol.* 50:350-359). In particular, the Examiner is of the opinion that Griffiths *et al.* teach

> the incubation of recombinant MetAP2 with ovalicin followed by incubation with florescein-fumagillin analog. The samples were dialyzed, alkylated, digested and subjected to HPLC separation. The absorbance of each eluate was monitored and the fractions corresponding to peaks for the binding of the florescein-fumagillin analog to the MetAP2 were collected (p15184, identification of the covalently modified MetAP2 residue by using florescein-fumagillin). It is disclosed that fumagillin and ovalicin covalently bind and inhibit MetAP2 (abstract). Griffiths *et al.* (*Proc. Natl. Acad. Sci., USA* **1998**, 95,15183-15188) does not disclose the administration of ovalicin, a fumagillin analog to a subject or removal of a biological sample from the subject.

The Examiner is also of the opinion that Morain *et al.* teach

> the assessment of pharmacodynamics for an inhibitor of prolyl endopepsidase (S 17092) (p350, method). The inhibitor was administered to patients and several parameters were measured, including the activity of prolyl endopepsidase in plasma. The collection of venous blood was measured for S17092 concentrations and assessment of plasma PEP activity (p351, protocol; p352, sample collection). Controls were used and it was concluded that (S 17092) had a potent dose-dependent inhibitory effect (p350, conclusion).

The Examiner then concludes that

> [a]t the time of the invention, it would have been obvious to one ordinarily skilled in the art to utilize the method to identify the binding inhibition of MetAP2 to/by a quantifiable irreversible inhibitor, such as a florescein-fumagillin, Griffiths *et al.* (*Proc. Natl. Acad. Sci., USA* **1998**, 95,15183-15188) where as Morain *et al.* (*Br. J. Clin. Pharmacol.* **2000**, 50, 350-359) discloses the administration of an inhibitor to patients and collecting blood to measure the inhibition. It is quite obvious to test a compound's properties in vivo after assessing their properties, such as binding or inhibition in vitro.

With respect to claims 19-25, cancellation of these claims has rendered the Examiner's rejection moot. With respect to claims 1 and 6 and claims dependent therefrom, and new claim 26, Applicants respectfully traverse the Examiner's assertion that the proposed combination of the above-cited references would have rendered the claimed invention obvious to the ordinarily skilled artisan at the time of the invention for the following reasons.

As discussed above, to establish a *prima facie* case of obviousness, it is necessary for the Examiner to present evidence, preferably in the form of some teaching, suggestion, incentive or inference in the applied references, or in the form of generally available knowledge, that one having ordinary skill in the art would have been motivated to make the claimed invention and would have had a reasonable expectation of success in making the claimed invention. Under section 103, "[b]oth the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure" (*Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.* 927 F.2d 1200, 1207, 18 USPQ2d 1016 (Fed. Cir. 1991), quoting *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed Cir. 1988)). Moreover, when a combination of references are used to establish a prima facie case of obviousness, the Examiner must present evidence that one having ordinary skill in the art would have been motivated to combine the teachings in the applied references in the proposed manner to arrive at the claimed invention. See, *e.g.*, *Carella v. Starlight Archery*, 804 F.2d 135, 231 USPQ 644 (Fed. Cir. 1986); and *Ashland Oil, Inc. v. Delta Resins and Refractories, Inc.*, 776 F.2d 281, 227 USPQ 657 (Fed. Cir. 1985). Finally, the prior art reference (or references when combined) must teach or suggest all of the claim limitations (M.P.E.P. 2143).

The primary reference relied upon by the Examiner, Griffiths *et al.*, teaches an *in vitro* assay to detect binding of MetAP2 to a fumagillin analog. However, Griffiths *et al.* fail to teach

or suggest ***administering a test compound to a subject,*** or using an irreversible quantifiable MetAP2 inhibitor recited in claim 26 to measure the amount of inactivated MetAP2, as required the pending claims. Moreover, Griffiths *et al.* fail to teach or suggest ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject***, as required by Applicants' claims. Furthermore, as evidenced by the Examiner's own admissions, there is no teaching or suggestion in Griffiths *et al.* to ***administer a test compound to a subject,*** or removing a biological sample from the subject, as required the pending claims. Thus, Griffiths, *et al.* fail to teach or suggest the claimed methods.

The teachings of Morain *et al.* fail to make up for the deficiencies of Griffiths *et al.* in that Morain *et al.* teach an *in vivo* assay in which a prolyl endopeptidase inhibitor is administered to a subject and ***a single biological sample from a single biocompartment*** is assayed for PEP activity. However, Morain *et al.* fail to teach or suggest ***removing a plurality of biological samples from the subject, wherein each of the plurality of biological samples is derived from a different tissue of the subject***, as required by the claimed methods.

In view of the foregoing, it is evident that, Griffiths *et al.* and Morain *et al.*, either alone or in combination, fail to teach or suggest the claimed invention and, thus, fail to render the claimed invention obvious.

Moreover, as discussed above, the Examiner has failed to point to any teaching in the cited references which would impel one of ordinary skill in the art to make the claimed invention. The mere fact that references can be combined or modified does not render the resultant combination obvious, unless the prior art also suggests the desirability of the combination. M.P.E.P 2143.01. The prior art must suggest "to those of ordinary skill in the art that they ***should*** make the claimed composition or device, or carry out the claimed process" and "[b]oth the suggestion and the reasonable expectation of success ***must be founded in the prior art, not in the applicant's disclosure*** (emphasis added)." *In re Dow Chemical Co.* 837 F.2d 469. 473, 5 U.S.P.Q.2d 1529, 1531 (Fed.Cir. 1988).

Thus, the mere fact that the teachings of Griffiths *et al. could* have been combined with the teachings of Morain *et al.* does not mean that the combination of the teachings would have been obvious to one of ordinary skill in the art at the time of Applicants' invention. Applicants

also submit that, as discussed above, transitioning from an *in vitro* assay format to an *in vivo* assay format is not as straightforward and simple as the Examiner presumes it to be, and thus one of skill in the art would not have had a reasonable expectation of success in arriving at the claimed *in vivo* methods.

In view of all of the foregoing, it is evident that the Examiner has failed to demonstrate that the motivation to combine the cited references existed at the time of the invention, and has also failed to demonstrate that the combined teachings of the cited art would have led one of skill in the art to the claimed invention. Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

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PATENT & TRADEMARK OFFICE

SUMMARY

Reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

The Commissioner is hereby authorized to charge any fees associated with the filing of this communication to our Deposit Account No. 12-0080, under Order No. PPI-144 from which the undersigned is authorized to draw.

Dated: April 13, 2007

Respectfully submitted,

By Maria Laccotripe

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Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew)

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Abstract

Dietary supplements are not subject to the same pre-market approval as conventional drugs, thus the true efficacy and, in cases, safety of these products is not known. The objective of this study was to evaluate the potential anti-inflammatory properties of three herbal constituents, apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew) on lipopolysaccaharide (LPS)-induced proinflammatory cytokine production, and to determine if effects in cell culture could predict results in an intact animal model. Murine macrophage cells and mice were treated with the stimulant LPS and herbal constituents, and resultant culture supernatant and serum, respectively, were evaluated for interleukin (IL)-6 and tumor necrosis factor (TNF)-α by ELISA. All three constituents inhibited LPS-induced IL-6 and/or TNF-α production in culture. Inhibition of these two cytokines was observed in mice, but did not display the same patterns of inhibition as cell culture data. The results suggest that all three constituents possessed anti-inflammatory properties, but that cell culture data can only be used to approximate potential effects in animals, and must be confirmed using appropriate animal models.

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Keywords: Apigenin; Chamomile; Ginsenoside; Ginseng; Parthenolide; Feverfew; Herbal; Cytokine; IL-6; TNF-α; LPS; Mouse

1. Introduction

Hundreds of herbal remedies have been used historically in maintenance of health and in the treatment of diseases. Today, such alternatives to over-the-counter medicines and prescriptions are still sought, with 36% of the US population using herbal products (McVean et al., 2000; O'Hara et al., 1998). The herbal supplement industry experienced tremendous growth throughout the 1990s with sales doubling every four years (Fleming, 1998) and projected sales expected to reach $6.6 billion by 2003 (Sloan, 2000). The overwhelming increase in herbal supplement sales has prompted concern over the need for appropriate regulations. Herbal supplements are legally defined as dietary supplements, and thus fall under the regulation of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Dietary supplements, unlike conventional drugs, do not require approval from the Food and Drug Administration (FDA) prior to marketing and sale of the product. Therefore, no investigations of the safety and efficacy of herbal products are required. Thus, it is often not known if these products are effective or even safe.

Abbreviations: alpha, α; minute, min; hour, h; DSHEA, Dietary Supplement Health & Education Act; FDA, Food & Drug Administration; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; COX, cyclooxygenase; PG, prostaglandin; ICAM, intercellular adhesion molecule; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ATCC, American Type Culture Collection; LT, leukotriene.

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One potential use for herbals is the treatment of inflammatory conditions. A number of inflammatory mediators are released by cells in response to localized injury or trauma. These mediators elicit or enhance particular functions of the inflammatory response and may be monitored to assess an inflammatory response. The primary inflammatory mediators are grouped into four main categories: chemokine, plasma enzyme, lipid and cytokine. Three herbal constituents, apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew), may possess anti-inflammatory properties. A limited amount of information now exists relating these herbal products, and their constituents, to specific anti-inflammatory mechanisms.

German chamomile, derived from *Matricaria recutita*, has been traditionally used as a popular folk remedy to treat digestive disorders, cramps, various skin conditions and minor infectious ailments (Tyler, 1993). Today, chamomile is most frequently used as a sleep aid and in the promotion of wound healing. Apigenin (4, 5, 7- trihydroxyflavone) is the best studied component of chamomile. This flavonoid has inhibitory effects on adhesion molecule expression (Panes et al., 1996; Wolle et al., 1996), prostaglandin (PG) E_2 (Liang et al., 1999; Panes et al., 1996), cyclooxygenase (COX)-2 (Liang et al., 1999) production and the proinflammatory cytokine interleukin (IL)-6 (Panes et al., 1996) in cell culture models.

The herbal supplement ginseng, derived from the roots of *Panax ginseng* or *Panax quinquefolium*, is estimated to be consumed regularly by more than 6 million Americans (The Lawrence Review, 1990). Ginseng extracts are frequently consumed for their ability to enhance physical performance and for their adaptogenic effects. These effects are believed to increase the body's ability to fight stress, increase resistance to disease by strengthening normal body function and as a result, reduce the detrimental effects of the aging process (O'Hara et al., 1998; Tyler, 1993). The ginsenosides are believed to be responsible for most of ginseng's activities (Attele et al., 1999; Fleming, 1998; Tyler, 1993; Wakabayashi et al., 1997). Cell culture studies have shown that ginsenoside Rb_1, one of the primary ginsenoside components (Bae et al., 2000), can inhibit lipopolysaccharide (LPS)-induced expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α (Cho et al., 1998). No in vivo data are available assessing the effect of ginseng on proinflammatory cytokine production.

Feverfew, *Tanacetum parthenium*, has been used for at least two millenia for the treatment of fever as well as headache, menstrual irregularities and stomachache (Tyler, 1993). Today, feverfew is widely used as a migraine preventative, and more recently as an aid for those suffering from arthritis (Jain & Kulkarni, 1999; Schinella et al., 1998; Williams et al., 1999). The majority of the medicinal properties are attributed to the sesquiterpene lactone parthenolide, which inhibits expression of intercellular adhesion molecule (ICAM)-1 (Dietrich et al., 1999), COX-2 and TNF-α production (Hwang et al., 1996) in vitro. No studies have been published which evaluated the effects of parthenolide on proinflammatory cytokine production in vivo.

Lipopolysaccharide (LPS), the major biologically active component of endotoxin, is derived from cell walls of gram-negative bacteria (Roth et al., 1997). LPS is a potent inducer of inflammation and can be used to induce production of inflammatory mediators, including proinflammatory cytokines, in model systems. Induction of the proinflammatory cytokines TNF-α and IL-6 by LPS can be monitored and evaluated to study the effect of pharmacological treatment, including herbals, on the inflammatory response. In this study, we hypothesized that apigenin, ginsenoside Rb_1 and parthenolide inhibit the expression of TNF-α and IL-6 in murine macrophage cell culture and intact animal model systems. Although all three constituents inhibited proinflammatory cytokine production in vitro, these results did not adequately predict effects in vivo.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. For in vitro studies, apigenin, ginsenoside Rb_1 and parthenolide (Aldrich, Milwaukee, WI) were dissolved in tissue culture grade dimethyl sulfoxide (DMSO) and further diluted in Dulbecco's modified Eagle's medium (DMEM, Sigma). The final concentration of DMSO in cell culture (less than 0.01% (v/v)) was not cytotoxic as determined by the colorimetric MTT (tetrazolium) assay (Mosmann, 1983). LPS from *Salmonella typhimurium* [1.5 EU/ng LPS] was diluted in DMEM. For in vivo studies, apigenin, ginsenoside Rb_1 and parthenolide were dissolved in DMSO; LPS was dissolved in endotoxin-free tissue culture grade water.

2.2. Experimental design for in vitro studies

The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in DMEM containing 4 mM L-glutamine, 3.7 g/l sodium bicarbonate and 4.5 g/l glucose. Medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 1% (v/v) NCTC-135 medium (Gibco BRL, Rockville, MD). Cells were maintained in a 5% CO_2 humidified incubator at 37 °C. Cell number and viability were assessed by trypan

blue dye exclusion using a hemacytometer (American Optical, Buffalo, NY).

RAW 264.7 cells (5×10^5 cells/ml) were cultured in flat-bottomed 48-well tissue culture plates (0.8 ml/well) (Corning, Corning, NY) with either apigenin, ginsenoside Rb_1 or parthenolide with or without stimulation by LPS. Supernatants were collected after 12, 24 and 48 h incubations (24 and 48 h data not shown) and stored at −80 °C until analysis.

2.3. Experimental design for in vivo studies

All animal handling was conducted in ordinance with guidelines established by the National Institutes of Health. Experiments were designed to minimize the numbers of animals used. Female B6C3F1 mice (8–10 weeks) were obtained from Charles River (Portage, MI). Animals were housed 3–4 per cage with a 12 h light/dark cycle, provided standard rodent chow and water *ad libitum*, and acclimated to their environment as least one week before the start of experiments.

In all cases, treatment with each herbal constituent was via p.o. administration to more closely approximate human exposure through consumption of herbal dietary supplements. Mice were pre-treated for 1 h with apigenin (50 mg/kg, p.o. in 50 μl) based on efficacious results obtained by Panes et al. (1996) and Mascolo et al. (1988) using similar treatment times and doses via i.p. administration. A 2 h pre-treatment with ginsenoside Rb_1 (25 mg/kg, p.o. in 50 μl) was used based on the previously reported kinetics of ginsenoside Rb_1, and its active metabolite, ginsenoside M1 (also known as compound K) (Wakabayashi et al., 1997), and treatment dose reported as efficacious by Jun-tain et al. (1990) and Hasegawa and Uchiyama (1998). Parthenolide (50 mg/kg, p.o. in 50 μl) was administered 1 h prior to LPS based on studies by Jain and Kulkarni (1999) and Mittra et al. (2000), and dose based on consideration of studies by Jain and Kulkarni (1999) and Schinella et al. (1998). LPS was administration at 1 mg/kg, i.p. in 100 μl. Vehicle-treated mice received 50 μl DMSO, p.o. and/or 100 μl H_2O, i.p. A 90 min time point sufficient to evaluate both IL-6 and TNF-α from one blood draw was chosen for sample collection based on time course data obtained by Zhou et al. (1999). Blood was obtained by retro-orbital bleeding and allowed to clot overnight at 4 °C. Serum was collected and analyzed for IL-6 and TNF-α by ELISA.

2.4. IL-6 and TNF-α determination by ELISA

Cytokine analysis for IL-6 (in vitro and in vivo) and TNF-α (in vitro) were performed using purified and biotin-conjugated rat anti-mouse IL-6 and TNF-α antibodies, respectively, from PharMingen (San Diego, CA). Streptavidin-peroxidase (Sigma) and 3,3′,5,5′-tetramethylbenzidine (TMB, Fluka, Ronkonkoma, NY) were used for detection. Absorbance was read at 450 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). For TNF-α analysis in vivo, the OptEIA Set: Mouse TNF-α (Mono/Poly) kit was used according to manufacturer's instructions.

2.5. Statistics

All statistical testing was performed using SigmaStat Statistical Analysis Software (Jandel Scientific, San Rafael, CA). For comparisons of multiple groups using parametric data, one-way analysis of variance (ANOVA) using Bonferroni's method were performed. For comparison of two groups, a Student's *t*-test was used.

3. Results

3.1. In vitro co-treatment with apigenin impairs LPS-induced IL-6, but not TNF-α production

To determine the potential inhibitory effects of herbal constituents on proinflammatory cytokine expression, RAW 264.7 murine macrophage cells were stimulated by LPS and co-treated with various herbal constituents. Apigenin, a flavone found in chamomile extracts and foods including parsley, bell peppers and guava was evaluated in vitro for potential inhibitory effects on LPS-induced IL-6 and TNF-α. RAW cells were co-treated with LPS and apigenin, 0.1–10 μg/ml, for 12 h. Apigenin doses evaluated were not cytotoxic as determined by the colorimetric MTT (tetrazolium) assay (results not shown) (Mosmann, 1983). LPS at 100 and 1000 ng/ml significantly induced IL-6 and TNF-α production following incubation (Fig. 1). No direct induction of proinflammatory cytokines was observed in vehicle or apigenin-only treated cells. Co-treatment with the two highest doses, 1 and 10 μg/ml (3.7 and 37 μM, respectively), of apigenin significantly, and dose-dependently, impaired LPS-induced IL-6. Significant inhibition of LPS-induced TNF-α was not observed.

3.2. In vitro co-treatment with ginsenoside Rb_1 dose-dependently inhibits LPS-induced IL-6 and TNF-α production

Ginsenoside Rb_1 is one of 12 major ginsenosides derived from *Panax* species. To test ginsenoside Rb_1's capacity to inhibit LPS-induced IL-6 and TNF-α production, RAW cells were co-treated with LPS and ginsenoside Rb_1, 0.1 to 100 μg/ml. No dose of ginsenoside Rb_1 tested caused cytotoxicity as determined by the MTT assay (results not shown). Significant production of IL-6 and TNF-α was observed following treatment

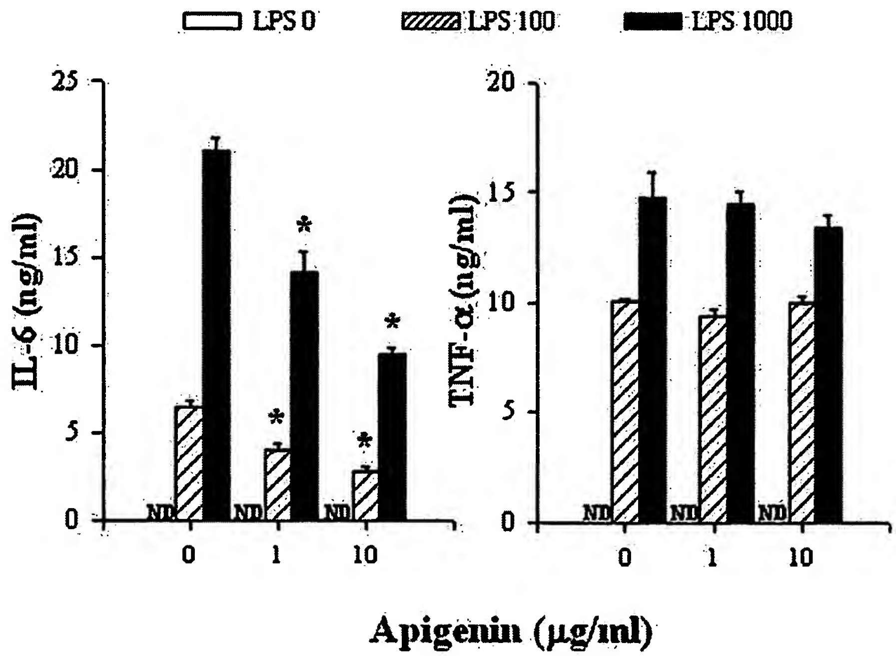

Fig. 1. Apigenin inhibits LPS-induced interleukin-6 (IL-6) but not tumor necrosis factor-alpha (TNF-α) production by RAW 264.7 cells. Cells (5×10^5 cells/ml) were cultured with LPS (0, 100 or 1000 ng/ml) in the presence or absence of apigenin for 12 h and supernatant analyzed for IL-6 and TNF-α. Data are mean±SEM ($n=6$) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($P<0.05$). ND indicates nondetectable.

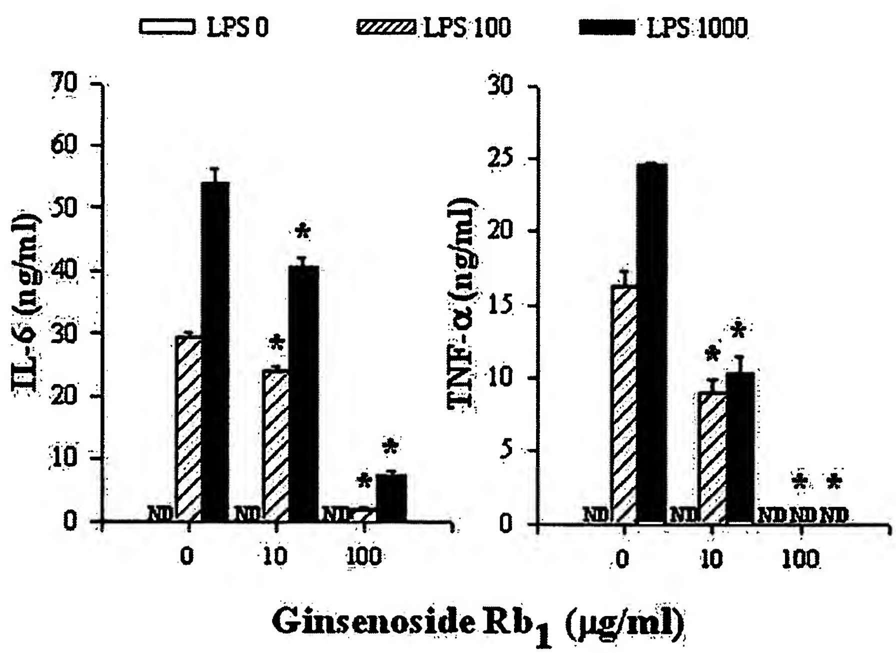

Fig. 2. Ginsenoside Rb_1 inhibits LPS-induced IL-6 and TNF-α production by RAW 264.7 cells. Cells were cultured as described in Fig. 1 with the presence or absence of ginsenoside Rb_1 and supernatant analyzed for IL-6 and TNF-α. Data are mean±SEM ($n=6$) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($P<0.05$). ND indicates nondetectable.

with 100 and 1000 ng/ml LPS (Fig. 2). No direct induction of IL-6 or TNF-α was observed in vehicle or ginsenoside Rb_1-only treated cells. Ginsenoside Rb_1 co-treated cells produced significantly, and dose-dependently, less LPS-induced IL-6 following 12 h incubation. Similar dose-dependent impairment of TNF-α was observed, with significant decreases in LPS-induced TNF-α following 10 μg/ml (8.4 μM) co-treatment, and a complete inhibition in cells co-treated with 100 or 1000 ng/ml LPS and 100 μg/ml (84 μM) of ginsenoside Rb_1.

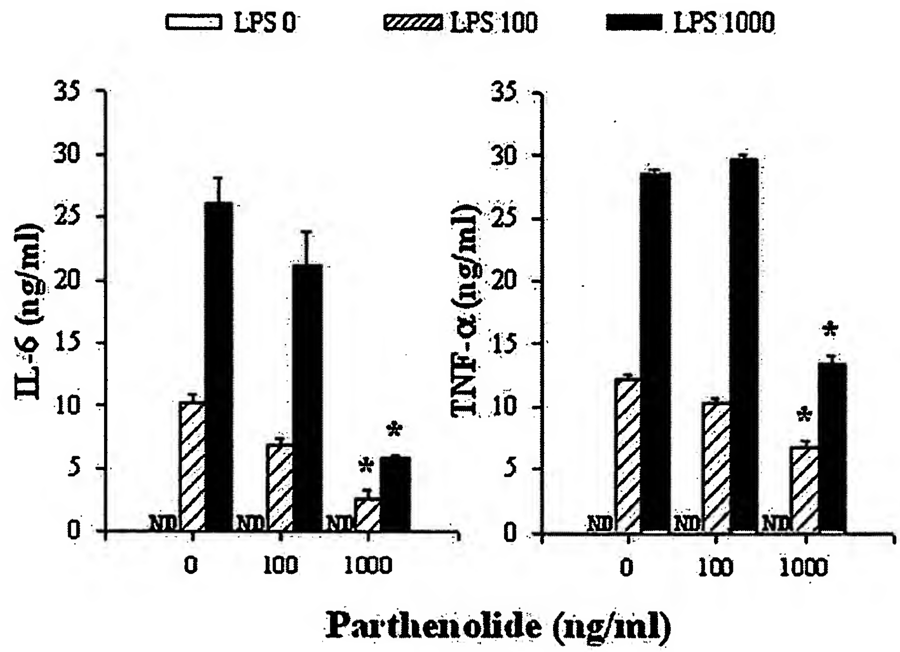

Fig. 3. Parthenolide inhibits LPS-induced IL-6 and TNF-α production by RAW 264.7 cells. Cells were cultured as described in Fig. 1 with the presence or absence of parthenolide and supernatant analyzed for IL-6 and TNF-α. Data are mean±SEM ($n=6$) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($P<0.05$). ND indicates nondetectable.

3.3. In vitro co-treatment with parthenolide inhibits LPS-induced IL-6 and TNF-α

Parthenolide, used as the constituent for standardization in commercial feverfew products, exhibits a wide variety of anti-inflammatory effects in murine and human cell systems. To determine potential effects on proinflammatory cytokine secretion, RAW cells were co-treated with LPS and 1–1000 ng/ml parthenolide. Cytotoxicity, as determined by the MTT assay, was not observed with parthenolide test doses (results not shown). Significant production of IL-6 and TNF-α was observed following 12 h exposure to LPS (Fig. 3). No direct induction of proinflammatory cytokines was observed in vehicle and parthenolide-only treated cells. Co-treatment with 1000 ng/ml (4 μM) parthenolide significantly impaired both LPS-induced IL-6 and TNF-α.

3.4. Apigenin pretreatment in vivo inhibits LPS-induced IL-6 and TNF-α

In order to more closely approximate potential human effects and to compare model systems, an animal model was employed using each herbal constituent and serum accumulation of LPS-induced proinflammatory cytokines as efficacy endpoint. Female mice were pretreated with apigenin (50 mg/kg, p.o.) based on previous in vivo studies by Panes et al. (1996) and Mascolo et al. (1988). After 1 h, LPS (1 mg/kg, i.p.) was administered and blood was collected 90 min later. Analysis of the serum showed significant levels of LPS-induced IL-6 and TNF-α production in controls (Fig. 4). Serum levels of IL-6 and TNF-α were not detectable in vehicle and apigenin control animals. One hour pretreatment with apigenin caused a significant reduction in LPS-induced proinflammatory cytokine production. LPS-induced IL-6 (Fig. 4A) was significantly reduced (35%), while TNF-α (Fig. 4B) was significantly reduced (33%) compared to animals treated with vehicle and LPS alone.

3.5. Ginsenoside Rb_1 pretreatment in vivo selectively inhibits LPS-induced TNF-α

The in vivo effects of ginsenoside Rb_1 on LPS-induced IL-6 and TNF-α levels in serum were also evaluated. Ginsenoside Rb_1 (25 mg/kg, p.o.) was administered 2 h prior to LPS (1 mg/kg, i.p.) based on the previously reported kinetics of ginsenoside Rb_1, and its active metabolite, ginsenoside M1 or compound K, in the sera of treated mice (Wakabayashi et al., 1997). Analysis of the serum following 90 min exposure to LPS showed significant induction of IL-6 and TNF-α, whereas these cytokines were unaffected in vehicle and ginsenoside Rb_1 control-treated animals (Fig. 5). No changes in IL-6 production (Fig. 5A) were observed with ginsenoside Rb_1 pretreatment. However, TNF-α production was significantly inhibited (Fig. 5B), (37%), by ginsenoside Rb_1 pretreatment.

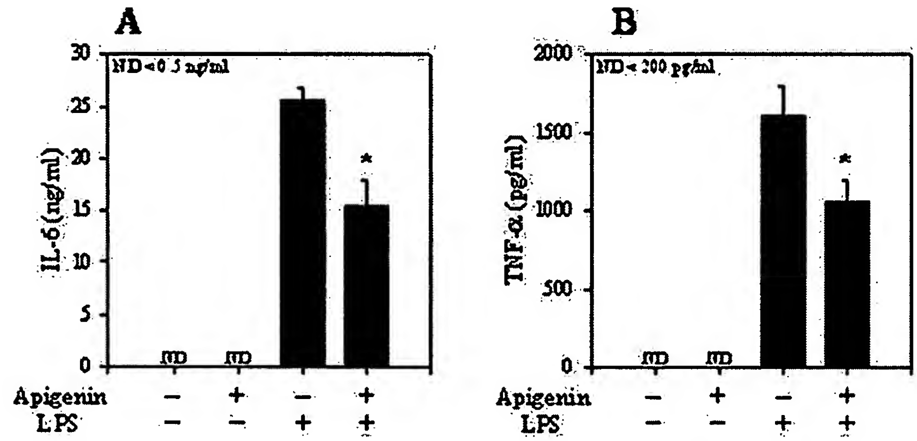

Fig. 4. Apigenin pretreatment in vivo inhibits LPS-induced IL-6 and TNF-α production. Female B6C3F1 mice were pretreated with apigenin (50 mg/kg, gavage) or vehicle (50 μl DMSO) for 1 h. LPS (1 mg/kg, i.p.) or vehicle (100 μl sterile water) was administered, and after 90 min blood was collected and serum analyzed for (A) IL-6 and (B) TNF-α. Data are mean±SEM ($n=12$, controls $n=6$). Values marked with an asterisk (*) differ significantly from LPS control values ($P<0.05$). ND indicates nondetectable. Data are a combination of three separate experiments.

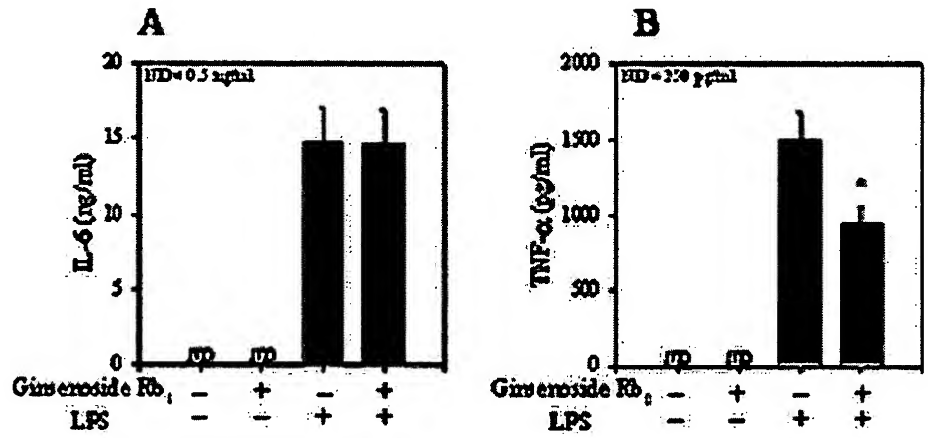

Fig. 5. Ginsenoside Rb_1 pretreatment in vivo inhibits LPS-induced TNF-α but not IL-6 production. Female B6C3F1 mice were pretreated with ginsenoside Rb_1 (25 mg/kg, gavage) or vehicle (50 μl DMSO) for 2 h. LPS (1 mg/kg, i.p.) or vehicle (100 μl sterile water) was administered, and after 90 min blood was collected and serum analyzed for (A) IL-6 and (B) TNF-α. Data are mean±SEM ($n=12$, controls $n=6$). Values marked with an asterisk (*) differ significantly from LPS control values ($P<0.05$). ND indicates nondetectable. Data are a combination of three separate experiments.

3.6. Parthenolide pretreatment in vivo shows no inhibitory effects on LPS-induced IL-6 and TNF-α

Parthenolide's ability to impair LPS-induced IL-6 and TNF-α was also evaluated in vivo. Mice were pretreated with parthenolide (50 mg/kg, p.o.) for 1 h prior to LPS (1 mg/kg, i.p.) administration. Serum was analyzed for IL-6 and TNF-α following a 90 min exposure to LPS. Serum levels of IL-6 and TNF-α were below detection in vehicle and parthenolide control-treated animals (Fig. 6). Although there was significant production of IL-6 and TNF-α by LPS, parthenolide pretreatment caused no significant changes in cytokine production for either IL-6 (Fig. 6A) or TNF-α (Fig. 6B).

4. Discussion

Consumer use of dietary supplements has grown significantly since the passage of the DSHEA in 1994 and herbals account for a considerable portion of total supplement sales (Blendon et al., 2001). Despite this growth, (Commission on Dietary Supplement Labels, 1998; Sarubin, 2000) there is still a considerable lack of scientific evidence to clearly support efficacy and, in some cases, safety of these products. The objective of this study was to establish a cell culture system that could be used to evaluate the potential efficacy of herbal constituents with presumed anti-inflammatory properties, and to determine if the results of cell culture studies

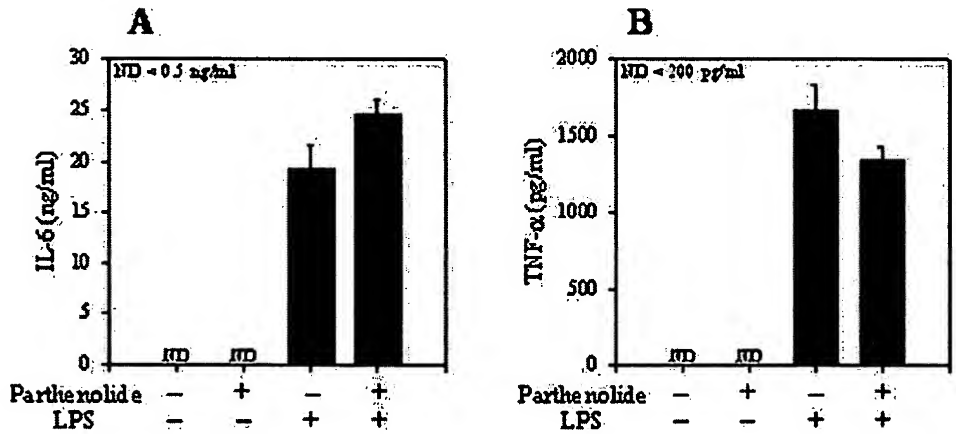

Fig. 6. Parthenolide pretreatment in vivo does not inhibit LPS-induced IL-6 and TNF-α production. Female B6C3F1 mice were pretreated with parthenolide (50 mg/kg, gavage) or vehicle (50 μl DMSO) for 1 h. LPS (1 mg/kg, i.p.) or vehicle (100 μl sterile water) was administered, and after 90 minutes blood was collected and serum analyzed for (A) IL-6 and (B) TNF-α. Data are mean±SEM ($n=12$, controls $n=6$). ND indicates non-detectable. Data are a combination of three separate experiments.

could accurately predict effects in an animal model. The potential anti-inflammatory properties of apigenin, ginsenoside Rb_1 and parthenolide were evaluated in RAW 264.7 cells because this clonal macrophage model produces high concentrations of IL-6 and TNF-α in culture upon activation with LPS, and reduced the need for additional animals required to obtain and use primary cultures. Cytokine levels were also evaluated in mice using LPS as a stimulant. The cytokines IL-6 and TNF-α were evaluated because these proinflammatory cytokines play a key role in the inflammatory response and can be easily quantified in supernatant and serum samples. Studies of apigenin's effect on proinflammatory cytokines are limited. The results of this in vitro study showed that apigenin significantly inhibited LPS-induced IL-6, but not TNF-α. Gerritsen et al. (1995) stimulated human endothelial cells with TNF-α and found that apigenin co-treatment inhibited IL-6 production as was observed herein for LPS treatment. However, in another study evaluating the inhibitory effects of apigenin on TNF-α production, Mastuda et al. (2002) showed that apigenin, at an $IC_{50}=5.3$ μM, inhibited antigen-IgE-mediated TNF-α secretion in RBL-2H3 mast cells. A potential reason for the differences in results may be the differences in stimuli and cell types used.

Although significant reductions in LPS-induced IL-6 by apigenin were observed here in cell culture, both IL-6 and TNF-α accumulation were inhibited in the animal model. No previous studies have evaluated the effect of apigenin on proinflammatory cytokine levels in vivo. Engelmann et al. (2002) assessed apigenin's in vitro and in vivo effects on cytostatic and anti-angiogenic action. The results showed effects in vitro, but lack of efficacy in vivo. However, animal studies evaluating the effect of apigenin on TNF-induced ICAM-1 expression (Panes et al., 1996) and carrageenan-induced PGE_2 and leukotriene (LT)B_4 (Mascolo et al., 1988) revealed inhibitory effects of apigenin similar to those observed in this study. The Panes study employed a 100 mg/kg dose and administered the apigenin i.p. 1 h prior to and 2 h following TNF-stimulation, while the Mascolo study employed a single 24 mg/kg, i.p. dose administered 15 min prior to the stimulus. The doses differed from the single 50 mg/kg, p.o. pretreatment employed in the present study, which was chosen as a result of observed anti-inflammatory effects in the above named studies. It is not clear how differences in mode of administration impact the results based on the specific doses evaluated in each of these studies since no evaluation of the pharmacokinetics of apigenin in mice are available. Additionally, because no reported levels of apigenin in chamomile products are available, it is unclear how the 50 mg/kg dose used in this study compares to reasonable human consumption through chamomile extracts.

Although ginsenoside compounds, including Rb_1, have been studied frequently relative to their metabolism (Akao et al., 1998; Attele et al., 1999; Chen et al., 1980; Hasegawa & Uchiyama, 1998; Wakabayashi et al., 1997), relatively few studies have evaluated the effect of ginsenoside Rb_1 on proinflammatory cytokine production in vitro and in vivo. The results of those latter studies are generally consistent with the findings herein. For example, Cho et al. (2001) showed that ginsenoside Rb_1 at an IC_{50} of 56.5 μM significantly impaired LPS-induced TNF-α in mouse and human macrophage cells. In another study that investigated the immunostimulatory activity of *Panax quinquefolius* (American ginseng) extracts containing ginsenoside compounds but no polysaccharides, significant TNF-α stimulating activity was observed (Assinewe et al., 2002). This effect, however, could not be induced using pure ginsenoside Rb_1. This finding agrees with our results showing that ginsenoside Rb_1 alone did not affect TNF-α levels in culture

supernatants and suggests that the immunostimulatory properties of the ginseng extract were not due to the presence of ginsenoside Rb_1, generally the ginsenoside in highest abundance in *P. quinquefolius* extracts (Harkey et al., 2001).

Although our in vitro data showed significant inhibitory effects of ginsenoside Rb_1 on LPS-induced IL-6 and TNF-α, our in vivo data did not follow the same pattern. Rather, oral gavage with ginsenoside Rb_1 inhibited LPS-induced TNF-α production but not IL-6 levels. Other studies evaluating ginsenosides in vivo support the anti-inflammatory claims attributed to ginsenoside Rb_1. A model of chronic inflammation in aged rats induced with multiple daily injections of LPS was used by Yu and Li (2000) to study the effect of ginseng root saponins (ginsenosides) on IL-1β and IL-6 mRNA expression. Daily treatment with 10, 20 or 40 mg/kg, i.p. ginseng root saponins markedly decreased the mRNA expression levels of both proinflammatory cytokines in the hippocampus compared to LPS-stimulated animals which did not receive ginseng root saponin treatment. These findings of IL-6-related effects contrast with our model, possibly because of the different route of exposure, target tissue and endpoint.

Compared to apigenin and ginsenoside Rb_1, many more studies have been undertaken to elucidate the anti-inflammatory properties of feverfew extract and parthenolide. For example, Hwang et al. (1996) showed LPS-stimulated TNF-α synthesis is inhibited by parthenolide in alveolar macrophage cells. Similarly, Uchi et al. (2002) showed that parthenolide impairs LPS-induced TNF-α production in human monocyte-derived dendritic cells. Additionally, parthenolide pre-incubation of HeLa cells prevents the induction of transcription from the IL-6 promoter (Bork et al., 1997). The results of previous in vitro studies are similar to our findings that parthenolide significantly impaired LPS-induced IL-6 and TNF-α production.

Although in vitro results showed here that parthenolide inhibited LPS-induced IL-6 and TNF-α, the in vivo findings were not in agreement. Only a limited amount of in vivo data are available for parthenolide, and specifically, no studies have been found in the literature which have evaluated its effects on proinflammatory cytokine responses in animals. However, other in vivo anti-inflammatory endpoints have been evaluated with respect to feverfew extracts and parthenolide in mice and rats (Jain & Kulkarni, 1999). When feverfew extracts from *Tanacetum parthenium* were administered by oral gavage, or pure parthenolide was injected i.p., both anti-inflammatory and antinociceptive effects were observed. Similarly, Tournier et al. (1999) showed that feverfew extracts and parthenolide from *Tanacetum vulgare*, administered by oral gavage, significantly reduced gastric ulcer index induced by ethanol in rats. Although these findings support the anti-inflammatory properties of parthenolide in animals, the anti-inflammatory effects could be the result of inhibition of multiple aspects of the inflammatory response. Other proinflammatory mediators including chemokines (MIP-2), plasma enzyme mediators (complement, kinin and clotting systems) and lipid mediators (COX, PG, platelet activating factor), may be affected rather than proinflammatory cytokines. This may explain the lack of effect observed here on the reduction of LPS-induced proinflammatory cytokine levels in the serum of parthenolide pretreated animals when compared to other anti-inflammatory studies using parthenolide.

In these studies, specific herbal constituents that are believed to be responsible for a majority of the extract's activity were chosen for evaluation. This was done to minimize variability that is frequently encountered in whole herb extract products (Harkey et al., 2001; Nelson et al., 2002). The intention was to use the results as a potential predictor of effects of whole herb extracts. Groenewegen and Heptinstall (1990), in a comparison of parthenolide and whole feverfew extracts on human platelet activity, showed that there are great similarities of feverfew extract and parthenolide on platelet aggregation and concluded, in platelets, that parthenolide from feverfew was responsible for the biological effects of the extract. In vivo studies by Tournier et al. (1999) showed similar effectiveness of parthenolide in comparison to whole extracts of feverfew in the cytoprotection of gastric mucosa against alcohol injury. However, Mittra et al. (2000) showed that parthenolide alone inhibited 5-hydroxytryptamine, but that it was evident the feverfew plant extract, which contains many other compounds, was more potent than parthenolide alone. These studies showed that focusing on specific individual constituents derived from herbal extracts may be used to predict the potential effect of a whole herb extract, but that additional, confirmatory studies using whole extracts need to be completed. These approaches may be used as a potential means of standardization of activities for parthenolide in feverfew and ginsenosides in ginseng.

Overall, the results of this study showed the compounds tested could inhibit proinflammatory cytokine responses. However, cell culture data did not always accurately predict the results in an animal model. This was supported by the findings that none of the three compounds evaluated displayed similar inhibitory activity in the cell and animal model systems for both IL-6 and TNF-α production levels. Discrepancies between cell culture data and animal data are not entirely surprising considering only one cell type exists in the culture studies, whereas multiple cell types capable of producing proinflammatory cytokines are present in a whole animal system. Additionally, other inflammatory mediators may influence the cytokine response. Finally, other key factors are metabolism and

distribution of the herbal constituents. Future perspectives should include understanding the molecular basis for inhibitory effects of these specific herbal constituents on proinflammatory cytokine gene expression and specifically the role of altered signal transduction.

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APPENDIX B

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Simulation of Human Luteal Endocrine Function with Granulosa Lutein Cell Culture*

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ABSTRACT

Human granulosa cells collected from *in vitro* fertilization have previously been cultured to provide a system to simulate the granulosa lutein cells of the corpus luteum. In most of these systems, the cultures have been relatively short term, and attempts to simulate the normal pattern of hormone production observed during the luteal phase of the cycle have not been reported. Additionally, the hormone relaxin has generally been absent from the endocrine analysis of these systems. In this report, methods were used that supported secretion of ovarian steroids and relaxin that mimics the profiles of these hormones *in vivo*.

This system was used to observe the endocrine responses of the granulosa lutein cells to three different protocols of CG administration designed to mimic the normal luteal phase, early pregnancy, and early pregnancy followed by pregnancy loss. The normal luteal phase was simulated by a constant baseline (0.02 IU/mL) CG model to simulate a nonconceptive cycle (baseline). The second model was baseline CG until day 8 of culture, followed by daily doubling from days 9–17 to simulate an early pregnancy (rescue-plateau). CG concentrations were then held constant from days 17–20 (5.12 IU/mL). A third model (rescue-drop) was used that was identical to the early pregnancy model except that on day 17 CG was returned to baseline concentrations (0.02 IU/mL) to simulate an early pregnancy loss.

Baseline CG stimulation resulted in profiles of estrogen, progesterone, and relaxin secretion in culture that were closely related to secretory profiles previously reported in serum during the nonconceptive luteal phase. The timing of appearance of relaxin secretion and later declines in steroid and relaxin secretion paralleled that observed in serum. In the CG rescue protocols, ovarian steroids rose in response to daily doubling of CG and fell when CG either plateaued or fell. Relaxin did not show an increase in response to increasing CG, but its secretion did not drop when CG concentrations plateaued or dropped. This cell culture system model mimics the profile of ovarian steroids and relaxin seen in serum during the nonconceptive luteal phase, although the relative magnitude of the hormones was not the same as seen *in vivo*. It was also used to investigate responses to luteal rescue protocols designed to simulate early pregnancy and pregnancy loss. This culture system may be useful to study differences in endocrine response in granulosa cells collected from different patients and to provide information of clinical relevance. This culture system provides a model to study luteal function and its response to different protocols of luteal rescue and thus may provide insight into early pregnancy and pregnancy loss. (*J Clin Endocrinol Metab* 82: 3078–3083, 1997)

HUMAN granulosa cells (GCs) recovered from *in vitro* fertilization (IVF) programs have been shown to luteinize in culture and thus have been used as a model to simulate granulosa lutein cells of the corpus luteum. However, the endocrine production from these cultures as generally conducted has not been compared with the *in vivo* luteal dynamics, and only rarely has the measurement of relaxin, a polypeptide produced by the corpus luteum, been included (1, 2). As measured by different groups, relaxin secretion has been undetectable during 24 days of culture (3), detected after a time lag of 14 or more days (1), or detected after 6 days of culture (2), whereas relaxin secretion *in vivo* is detected 5–7 days following the LH peak (4–6). In the one study in which relaxin secretion began at a time corresponding to *in vivo* timing (2), it was not followed past 9 days of culture, so its profile could not be compared with events later in the cycle.

Most *in vitro* culture systems for human GCs have used cells plated on plastic. However, the use of a proteinaceous matrix to keep cells from being in contact with the plastic has been used by some investigators (7, 8) to provide a more physiological environment. Human GCs grown on an extracellular matrix have been shown to have a higher production of progesterone (9, 10) and estradiol (10, 11), form more gap junctions with neighboring cells (9), have more LH/CG receptors (9), and have a greater cAMP response to CG (12) than cells grown on plastic. It has been suggested that a cell adhesion receptor (an integrin) and laminin and fibronectin play important roles in the differentiation of GCs to luteal cells in the rat (8). Laminin and fibronectin are major glycoprotein components of the extracellular matrix (Matrigel matrix, Becton-Dickinson Labware, Franklin Lakes, NJ). We have utilized this matrix to try to develop a granulosa lutein cell culture system that produces relaxin and ovarian steroids in a physiological manner.

CG is administered to the GCs to cause luteinization and maintain functionality of the granulosa lutein cells during culture. The common CG dose of 1 IU/mL appears to date from early studies when human IVF clinics first became a source of GCs, and the potential of this model was devel-

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oping. However, the literature does not contain a systematic study of responses to lower CG doses, and the use of 1 IU/mL may represent more CG than necessary to simulate a normal luteal phase in which gonadotropin support is low.

The human granulosa lutein cell culture system used in this study combined the use of an extracellular matrix, lower concentrations of CG than generally used by other investigators, and a sensitive relaxin assay. This system was used to simulate a normal luteal phase profile of steroids and relaxin. These profiles can be altered by the use of CG protocols to simulate early pregnancy and early pregnancy loss. This granulosa lutein cell model could prove useful to study patterns of early pregnancy loss by the design of different CG stimulation protocols that mimic patterns of CG observed *in vivo*. It could also be useful for analysis of GCs from assisted reproduction patients to determine whether abnormalities exist in their normal and CG-stimulated endocrine production.

Materials and Methods

Media and plate preparation

MEM (Gibco, Grand Island, NY) was modified with the following additions: sodium bicarbonate, 4.4 mg/100 mL MEM (Sigma, St. Louis, MO); fungizone, 1 mL/100 mL (Gibco); penicillin G, 6 mg/100 mL (Sigma); streptomycin sulfate, 6 mg/100 mL (Sigma), and 10% FCS (Hyclone, Logan, UT). Media was filtered through a 0.22-μm sterile syringe filter (Fisher, Santa Clara, CA) and equilibrated at 37 C and 5% CO_2 in air before use. CG (Pregnyl; Organon, West Orange, NJ) was added to the culture media in amounts as described below. Extracellular matrix was applied to culture dishes on the same day cells were collected according to the manufacturer's directions.

Cell collection

Human GCs were obtained by ultrasound-guided follicle aspiration from women receiving assisted reproduction treatment at Pacific Fertility Center (Sacramento, CA). The cells were a by-product of the IVF/embryo transfer (ET) procedure and are normally discarded. They were provided to this study as coded samples with the identities of the women unavailable, so this research was exempt from review by the university Human Subjects Review Committee (HSRC) under Federal exemption category number 4. Exemption from full HSRC review was approved by the HSRC coordinator. The patients received varying doses of Metrodin (Serono Laboratories, Randolph, MA) and Pergonal (Serono) and received 10,000 CG 36 h before follicular aspiration. Individual follicles were not distinguished, because all GCs from an individual were pooled. Cells from different subjects were not pooled.

Culture preparation

Cells were prepared by initial centrifugation followed by layering onto a 40% Percoll (Sigma). The GC layer was washed twice with 5–10 mL fresh MEM and centrifuged for 10 min at 300 $\times$ *g*. The supernatant was discarded, and the pellet was resuspended in 2–4 mL MEM. GCs were filtered through an 89-μm polyester filter (Spectra/Mesh, Spectrum Medical, Laguna Hills, CA) just before being counted and plated. Cells were brought to a final concentration of 1×10^5 cells/mL in MEM and plated on 4-well plates (1.9 cm diameter wells) at 5×10^4 cells/well (0.5 mL). Cells had attached after 24 h, and media was changed to remove remaining debris. Media was changed daily in all experiments and stored frozen until assay for hormone concentrations. Because multiple wells were obtained from each subject, different wells from each subject were used for the various treatment protocols (viability and either CG dose response or CG stimulation).

Verification of viability and cell number during culture

Estimates of viability were obtained using trypan blue (0.4%, Gibco) exclusion on an Olympus CK2 microscope (Olympus Optical, Tokyo, Japan) at 200 times magnification. One milliliter Matrisperse (Fisher) was added to each well to free cells from the Matrigel, and cells were scraped into a centrifuge tube. The well was rinsed with an additional 1 mL Matrisperse, which was placed in the tube and kept on ice for 1 h. Cells were centrifuged for 5 min at 500 $\times$ *g*, and the pellet was resuspended in 100 μL PBS. Cells were counted on a hemacytometer.

CG dose response

To determine the lowest dose of CG needed to effectively maintain luteal support in terms of steroid and relaxin secretion, GCs collected from six patients were plated and cultured with seven different constant concentrations of CG. The doses of CG ranged from 0.002–0.2 IU/mL culture fluid, and these doses were maintained throughout the 20-day culture period. Estradiol, progesterone, and relaxin concentrations were determined in the conditioned media.

CG stimulation protocols

Three protocols of CG administration to the culture media were used to simulate three different luteal phase events using replicate wells from 10 subjects. The first protocol (baseline) was a constant baseline dose of CG to simulate a normal nonconceptive luteal phase. A baseline concentration of 0.02 IU/mL was selected from the dose-response study based on its ability to maintain physiological profiles of steroid and relaxin secretion. CG concentrations were held at baseline CG for each of the 20 days of culture. A second protocol (rescue-plateau) was used to simulate early pregnancy during the middle of the culture period. CG was maintained at baseline concentrations (0.02 IU/mL) for days 1–8 of culture and were then doubled each day until day 16 of culture. On days 17–20 of culture, CG concentrations were maintained at the highest CG concentrations (5.12 IU/mL) to determine the effect of plateaued CG concentrations for comparison with the third protocol. The third protocol (rescue-drop) was designed to simulate an early pregnancy followed by pregnancy loss. In this protocol, CG concentrations were identical to the previous protocol until day 16. On day 17 and thereafter CG concentrations were returned to baseline (0.02 IU/mL).

Assays

Estradiol and progesterone were measured by commercial kits (Diagnostic Products Corp., Los Angeles, Ca) as previously reported (13). Relaxin was measured by an enzyme immunoassay as previously reported for serum relaxin (5). The assay was modified by dilution of human relaxin using culture fluid instead of human serum for preparation of standards.

Data analysis

To normalize the endocrine data, the values were converted to the common logarithm for statistical analysis and averaging. Data were converted to arithmetic scale for graphing (geometric mean). Hormone values for the three CG protocols were compared by two-way repeated measures ANOVA, and significance was followed up by Student-Newman-Keuls multiple comparisons test using a 0.05 significance level.

Results

Verification of cell number and viability during culture

Cell number was verified on day 16 of culture (n = 6 patients). Mean cell counts per well were 50,000 $\pm$ 10,488 and 52,000 $\pm$ 12,000 for CG baseline and CG rescue protocols, respectively. Day 16 viability ranged from 95–98%.

Endocrine responses

In analysis of the results of CG dose response and CG stimulation protocols, cells from different patients resulted in two distinct patterns of relaxin response (not shown). The first, termed a nonresponder, was characterized low relaxin production. The second pattern, termed a responder, was

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characterized by robust relaxin concentrations on day 10 of culture. A responder was defined as a patient with >150 pg/mL relaxin on day 10 of culture using the standard baseline dose of CG (0.02 IU/mL). There was a clear distinction between responders and nonresponders, because the highest relaxin concentration from a nonresponder on day 10 was 46 pg/mL and the lowest relaxin concentration from a responder was 464 pg/mL. Four of six patients in the CG dose-response protocols and six of ten patients in the CG stimulation protocols were responders, and only the results from these were used for further analysis.

CG dose response

The cells showed a dose-response relationship to CG in terms of steroid and relaxin production with the CG concentrations used in this study (Fig. 1). A dose of CG of 0.02 IU/mL was chosen as the standard baseline dose of CG. This dose gave adequate steroid and relaxin production as well as a timely profile of relaxin secretion. Higher amounts of CG tended to give prolonged relaxin secretion past day 15 of culture, and thus might indicate that the CG concentrations were higher than desired to simulate a nonconceptive cycle.

CG stimulation protocols

Estradiol concentrations produced by the baseline CG protocol cells were extremely high on the first day of culture (data not shown). Concentrations significantly declined on days 3 and 5 of culture (Fig. 2), increased from days 5–11, and then declined between days 11–19 ($P < 0.05$). Progesterone secretion significantly increased on alternate days from days 1–7 of culture. Progesterone then declined between days 7–11, with a significant drop between days 15–17 of culture. Relaxin secretion was first significantly elevated on day 5 of culture and then showed significant daily increases through day 8. Relaxin levels plateaued on day 9 and remained elevated until day 15 of culture. There was a significant fall in relaxin secretion from days 15–18 and thereafter.

Estradiol concentrations from cells treated with the CG rescue-plateau protocol (Fig. 3) were similar to those from baseline CG cells before the increase in CG. During the period of culture that the CG was increasing, estradiol concentrations were prevented from dropping. There was a significant increase in estradiol from days 9–11 and 13–15 of culture. Estradiol concentrations began a significant drop from days 15–19 of culture, during the time CG concentrations were plateaued. Progesterone concentrations followed a nearly identical profile to estradiol concentrations in response to the increase in CG. There was a significant increase in progesterone from days 9–11 and 13–15 of culture. Plateaued CG resulted in a significant drop from days 15–17 and 19 of culture. Before day 16 of culture, relaxin concentrations in the rescue-plateau protocol were not different from the baseline protocol. However, the increasing concentrations of CG prevented the decline of relaxin, because days 16–20 were not different from day 15 of culture.

In the rescue-drop CG protocol (Fig. 4), estradiol, progesterone, and relaxin concentrations and profiles were not different from the rescue-plateau protocol.

A comparison of the hormones from the three CG stimulation protocols is presented in Fig. 5. The divergence of estradiol concentrations in the two CG rescue protocols from the concentrations in the baseline protocol was significant on day 13 and thereafter. A difference was noted for proges-

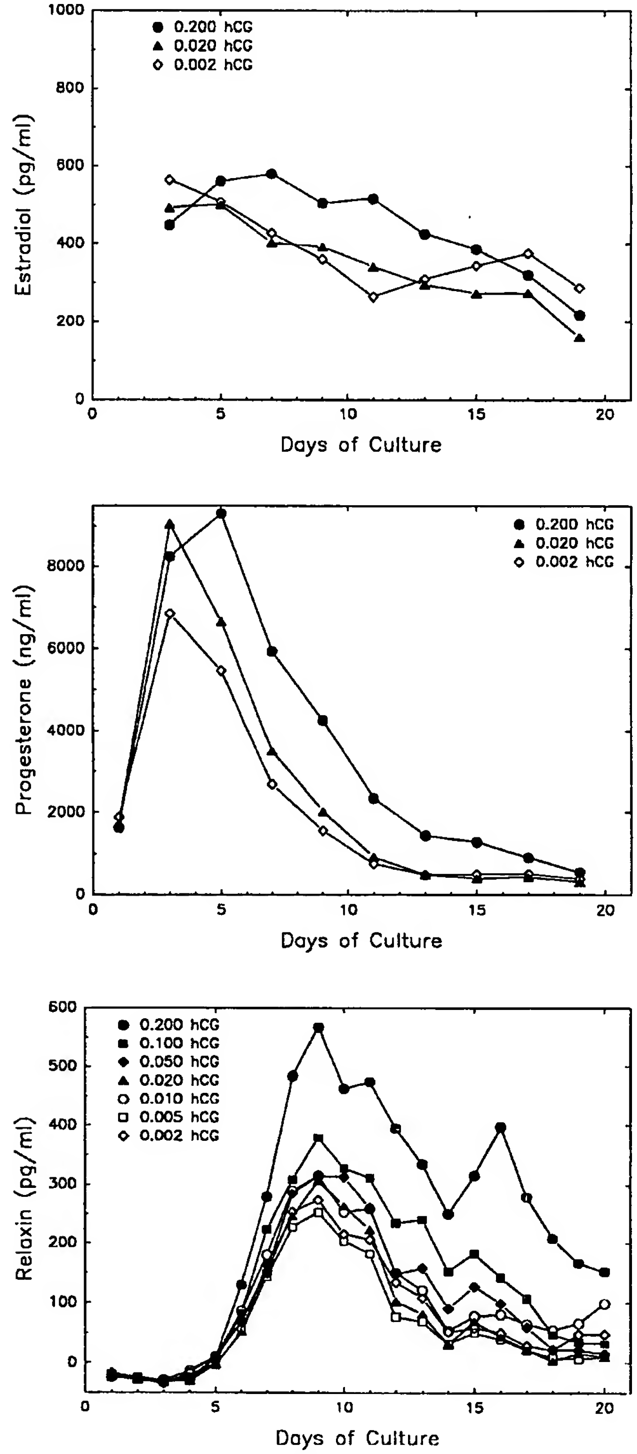

FIG. 1. Mean estradiol, progesterone, and relaxin concentrations from four subjects treated with constant amounts of CG throughout culture period at doses shown (in international units per milliliter).

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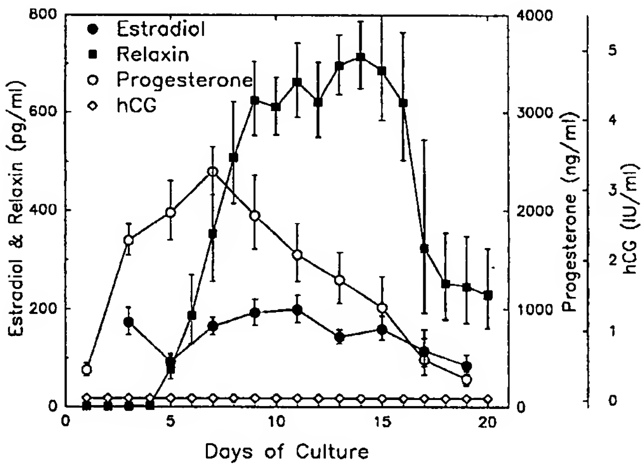

FIG. 2. Geometric mean estradiol, relaxin, and progesterone profiles from GC cultures treated with baseline CG protocol (n = 6 subjects).

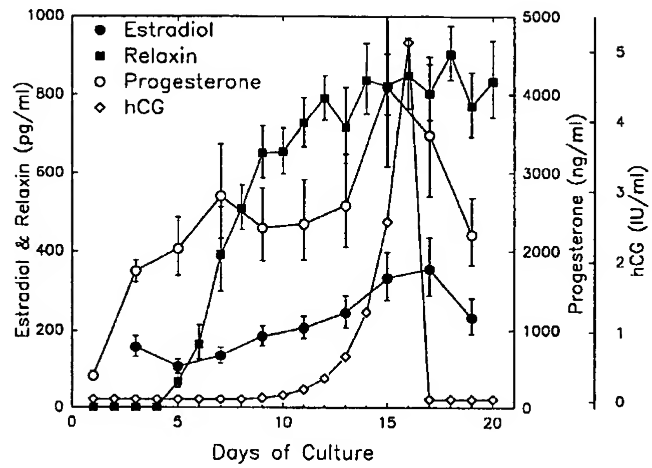

FIG. 4. Geometric mean estradiol, relaxin, and progesterone profiles from GC cultures with rescue-drop CG protocol (n = 6 subjects).

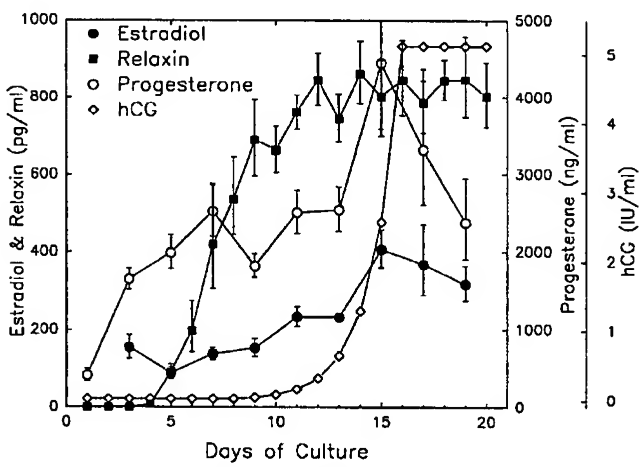

FIG. 3. Geometric mean estradiol, relaxin, and progesterone profiles from GC cultures treated with rescue-plateau CG protocol (n = 6 subjects).

terone concentrations on day 11 from rescue protocols compared with the baseline protocol, but this difference was not significant until day 13 of culture. The difference in relaxin concentrations between CG baseline and the two rescue protocols was significantly different after day 16.

Discussion

Human GCs grown in culture using Matrigel and maintained on a low (0.02 IU/mL) chronic dose of CG were used to simulate the profiles of steroid and relaxin concentrations similar to that observed in a normal human luteal phase in which conception is absent. The *in vitro* endocrine profiles obtained on this protocol (Fig. 2) are similar in shape and timing to our *in vivo* profiles of steroids (14) and relaxin (4, 6) from women with natural cycles sampled on a daily basis. This is the first report of granulosa lutein cell culture in which relaxin secretion mimics that observed in the normal luteal phase, and suggests that this is a useful model for the study of the control of luteal function. Although one other group has detected relaxin secretion in culture as early as was observed in this study (2), they only reported data for 9 days of culture, so long-term profiles were not provided. The absence of the appearance of relaxin secretion before day 5 of culture is consistent with the absence of circulating relaxin early in the luteal phase (4) and the absence of relaxin messenger RNA in the early luteal phase corpus luteum (15). The reason that relaxin is not immediately produced from the beginning of culture or early in the luteal phase of the cycle is not known. The finding of a similar delay in culture compared with that observed *in vivo* suggests that this is an inherent feature of granulosa lutein cells and not caused by the influence of factors arising outside the ovary.

Under these culture conditions, two types of endocrine patterns were observed, one termed nonresponder because of an absence or minimal relaxin secretion and the other termed a responder because of a more robust relaxin response. Although steroid responses tended to be lower in nonresponders, they were not as strikingly different as relaxin concentrations, which were well separated in these two groups. It is not known whether a larger number of subjects would give a continuum in terms of relaxin secretion or whether these responses represent two distinct groups of subjects. Mayerhofer *et al.* (2) did not report on variations in relaxin secretion between patients, so it is not known whether this was observed in their system. Gagliardi *et al.* (1) reported a wide variation in the days of culture before relaxin was detected (10–22 days), but once secretion began 9 of 10 patients had relaxin production >200 pg/mL. The reason we saw 37% nonresponders (6/16) in the CG dose-response and CG stimulation protocols may because of the much smaller basal amounts of CG used in this culture system. We are continuing to collect information from nonresponders and are actively investigating the possible reasons for differences in relaxin secretion of GCs collected from different patients. It is speculated that endocrine production *in vitro* may be related to *in vivo* endocrine production. We have shown that serum luteal phase relaxin concentrations are highly variable between subjects, more so than progesterone concentrations (16). It was also found that relaxin concentrations were better

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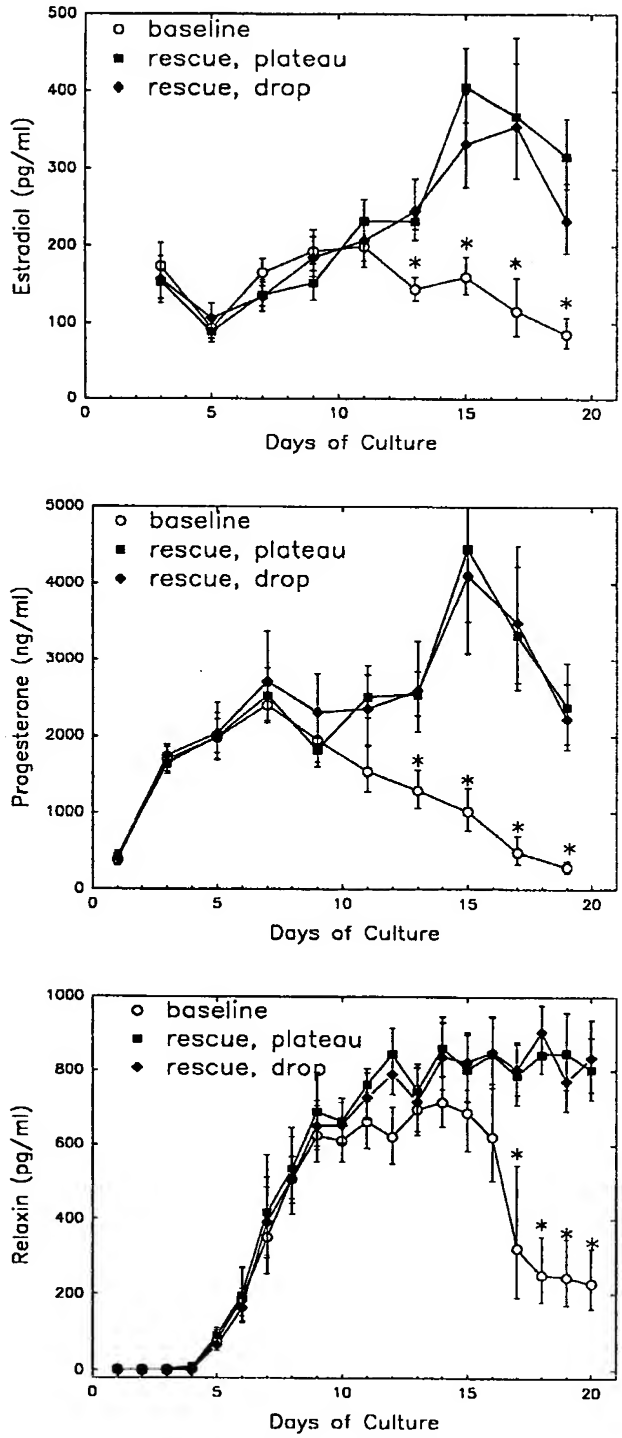

FIG. 5. Geometric mean estradiol, progesterone and relaxin from granulosa lutein cell culture in response to stimulation with three models (n = 6 subjects). Rescue-plateau and rescue-drop groups were not significantly different. The *asterisk* (*) indicates significant difference of baseline from rescue-plateau and rescue-drop groups.

correlated with the stage of the endometrial biopsy than progesterone (16), so relaxin concentrations may have physiological relevance. We are currently investigating the relationship of the profile of endocrine production in culture with serum values of these hormones in the same cycle from which cells were collected.

The decline in endocrine function during the latter days of culture is similar to the decline in luteal function late in the cycle. This may indicate an inherent pattern of endocrine secretion in the granulosa lutein cells, because this secretion pattern was in response to constant CG concentrations. Because cell number and viability were not changing, the change in endocrine concentrations must reflect different rates of endocrine production and secretion. The cell number did not appear to change during the 20 days of culture for human granulosa lutein cells grown on Matrigel. This is unlike GCs grown on plastic, which can double after 3 days in culture (17) or multiply 2- to 7-fold during a 24-day culture (3). It is possible that Matrigel contains some growth inhibitory factors that would be absent for cells grown on plastic, but if these factors were soluble they would be rapidly removed by the daily changes in culture fluid. Viability remained high throughout the culture period and thus did not appear to be the reason for differences in hormone production in response to CG.

There were at least two major differences between steroid and relaxin secretion in response to CG that were observed in these cell cultures. The first difference was that daily doubling of CG concentrations resulted in significantly increased steroid but not relaxin concentrations. The only significant effect of increasing CG on relaxin concentrations was prolonged secretion. This is different from the response of relaxin to CG *in vivo*, in which relaxin increases rapidly in parallel with trophoblastic CG in early pregnancy (5) or to the administration of exogenous CG in the nonconceptive luteal phase (18). Others have observed significant increases in relaxin in culture in response to an increase in CG, but the dose was 100 IU/mL (2), which probably represents a nonphysiological increase. It may be that the increase in CG used in this study was too gradual to give a significant increase in the amount of relaxin secreted. There appeared to be a small, although nonsignificant, increase in relaxin secretion in response to CG. A more vigorous rise in CG may be required to enhance relaxin secretion, and we are experimenting with different gradients of CG to test this. Alternately, there may be other factors in early pregnancy, in addition to CG, that also stimulate relaxin secretion but are absent from this culture system.

The second difference between granulosa lutein cell production of steroids and relaxin was in response to plateaued or dropping CG concentrations. A halt to daily doubling of CG, either in the rescue-plateau or rescue-drop protocol, resulted in an immediate fall in both estradiol and progesterone concentrations. The profile of steroids was contrasted by that of relaxin, which remained elevated with either a plateau or a drop in gonadotropin support. Thus, the granulosa lutein cells appear to require continually increasing CG to maintain steroid secretion, whereas they only require the prior elevation of CG above baseline to maintain enhanced relaxin secretion. The fall in steroid concentrations, but not relaxin, in response to plateaued CG concentrations is similar to profiles of circulating steroids and relaxin observed in women with early pregnancy loss (5). In cases of early preg-

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nancy loss, CG can be observed to plateau and then fall. As observed *in vitro*, circulating steroids began to fall immediately as soon as CG concentrations plateaued, whereas relaxin concentrations remained elevated as long as CG was present in circulation (5). The differences between the steroid and relaxin response to CG could have several causes. There may be a differential sensitivity of the relaxin and steroid synthetic machinery to CG stimulation. Although the initial pathways for CG response are through the CG receptor and second-messenger systems, later events in their stimulation may have different regulation and responsiveness. Alternately, different granulosa lutein cells may have different sensitivities to CG stimulation and differential production of steroids and relaxin. It is possible that some cells produce steroids, whereas others produce relaxin, each with a different responsiveness to CG.

This GC culture system may be useful for the study of the cell types that produce relaxin and ovarian steroids and the control of endocrine secretion. This system produces steroid and protein markers with timing and profiles similar to that seen *in vivo* and allows the application of CG rescue protocols to be explored. With the ability to rescue the cell culture, much as the corpus luteum of early pregnancy is rescued, this system provides the ability to study the effects of different CG protocols on luteal function. This system might also prove useful for the study of altered forms of CG, which have been implicated in early fetal loss (19).

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APPENDIX C

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FORUM

Bridging the Gap between *in Vitro* and *in Vivo* Models for Neurotoxicology

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In vitro systems are widely used for investigation of neurotoxicant-induced perturbations of cellular functions. A variety of systems exist that demonstrate certain similarities to neurotoxicant-induced events in the intact animal are discussed, including single-cell types, systems that consider endpoints relevant in toxicology, and systems that consider heterogeneous cell interactions. Relationships between the *in vitro* and *in vivo* systems are examined in which ethanol, lead, polychlorinated biphenyl compounds, and organophosphate insecticides are examples. Situations in which the *in vitro* systems have been used to advantage are provided, along with cautions associated with their use.

Key Words: neurotoxicity; *in vitro* models; continuous cell lines; heterogeneous cell systems; dosing regimens; nervous system development and maturation.

This commentary addresses recent advances in the development and use of *in vitro* systems for the study of neurotoxicants, focusing on the validity of such models when compared to available *in vivo* data. Bridging the *in vivo/in vitro* gap was the topic of a poster discussion session at the 1998 Society of Toxicology meeting. At that time, approaches and problems associated with integrating *in vitro* and *in vivo* data were discussed. The *in vitro* systems used by the investigators included, in order of increasing complexity, crude cortical membranes (Lasley and Gilbert, 1998), continuous cell lines (Ehrich and Correll, 1998; Tiffany-Castiglioni *et al.*, 1998), primary cultures of rat astrocytes (Costa *et al.*, 1998; Tiffany-Castiglioni *et al.*, 1998), rat cerebellar granule neurons (Kodavanti, 1998), primary motor neurons of dissociated cultures of murine spinal cord (Durham *et al.*, 1998; Roy *et al.*, 1998), rat brain region organ cultures (Dees *et al.*, 1998; van den Beukel *et al.*, 1998), and hippocampal slices of exposed animals used *ex vivo* (Lasley and Gilbert, 1998). In addition, several investigators used systems which had been genetically altered to express certain proteins, i.e., enzymes or receptors (van den Beukel *et al.*, 1998; Durham *et al.*, 1997, 1998; Roy *et al.*, 1998). Regardless of the system used for study, one topic threaded through the discussion, the parallel between whole animal (*in vivo*) models and models that did not use whole animals, such as *ex vivo* models (tissues from exposed animals) or *in vitro* models (cells and tissues that did not involve exposure of animals). Also discussed were validation of cell culture observations and improvement of *in vitro* models. These topics are considered in the context of specific dilemmas that the authors have addressed in their work. This includes the interpretation of various *in vitro/ex vivo* experimental systems, especially endpoints indicative of toxicities that occurred under specific dosing regimens, with comparison to *in vivo* models. The authors also discuss the judicious use of immortalized cell lines (developmental phenotype, validity, and extension to more complex models), and the constructive interplay or reconciliation of *in vitro* and *in vivo* approaches. The *in vitro* systems were used to study the toxicities of ethanol, lead (Pb), antiesterase compounds, and polychlorinated biphenyls. The spinal cord motor neuron culture was used to investigate mechanisms

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associated with a neurodenerative disease, amyotrophic lateral sclerosis (Durham *et al.*, 1997; 1998; Roy *et al.*, 1998).

The objective of neurotoxicologic studies on cells and tissues *in vitro* is to characterize the cellular and molecular substrates and pathways that contribute to impaired behavior, altered function, or pathological changes from exposure to a toxicant (Harry *et al.*, 1998). These substrates form a broad spectrum that reflects the hierarchy of biological organization. At one end of the spectrum is the cultured neural cell, and at the other end is an organism that exhibits behavior and mentation. In the middle is a formidable knowledge gap. Research with a number of neurotoxicants (such as organophosphates, halogenated aromatic hydrocarbons, ethanol, and heavy metals) is methodically proceeding from both ends of the spectrum, but so far without indication that the two shall meet. However, it should now be possible to take some bolder steps from the reductionist (molecular and cell biology) end into the uncharted middle ground, based on an improved understanding of the usefulness and limitations of several *in vitro* models.

Seeing the World in a Grain of Sand

Most mechanistic events within neurotoxicologic processes *in vivo* are remote from direct observation or manipulation by the investigator. Therefore, toxicologists devise reductionist biological models of putative target molecules, cells, or tissues involved in the processes. By exposing these models to toxicants and observing their responses, investigators infer corresponding responses *in vivo*. The "grain of sand" in which we attempt to see the world may be one protein of interest in a homogeneous cell line, an *ex vivo* tissue preparation from a toxicant-exposed animal, or any of several other models of intermediate complexity. Examination of the model can be remarkably illuminating or of limited value, depending on constraints imposed by the model itself, the manipulations or observations made, and the capacity for compensatory responses to extended exposure. In all cases, fidelity to the *in vivo* condition is a hallmark of the validity of *in vitro* reductionist models. Reductionist models of various levels of complexity are often helpful for delineating or substantiating cellular or molecular mechanisms that contribute to toxicant action, as they were when *in vitro* systems were used for study of ethanol, polychlorinated biphenyls, and organophosphorus insecticide toxicities (Barber *et al.*, 1998; Costa *et al.*, 1998; Dees *et al.*, 1998; Ehrich *et al.*, 1998; Kodavanti, 1998; van den Beukel *et al.*, 1998).

The participants at the poster discussion session examined several examples from their collective experiences that illustrate both successes and failures of *in vitro* approaches to neurotoxicity testing. Toxicant concentrations, intracellular targets, and the need for controls were discussed. With these considerations, *in vitro* models could be used to study in an isolated context the neurotoxic effects occurring *in vivo*. In a number of cases, these effects could occur at toxicant concentrations relevant *in vivo,* such as second messenger effects initiated by ethanol or polychlorinated biphenyls (Balduini *et al.*, 1991; Costa *et al.*, 1995, 1998; Kodavanti, 1998; Kovacs *et al.*, 1995) and esterase inhibition caused by protoxicant organophosphorus compounds activated by an inorganic catalyst or by microsomal enzymes (Barber *et al.*, 1998, 1999; Ehrich and Correll, 1998). Somewhat lower concentrations of ethanol were needed for *in vitro* induction of cell proliferation than for that *in vivo,* but both types of experiments indicated temporal windows of susceptibility that coincided with the peak of the brain growth spurt (Balduini *et al.*, 1991). However, even if the effects are similar with *in vitro* and *in vivo* systems, as they were with long-term potentiation following chronic exposure to lead (Lasley and Gilbert, 1998), the magnitude of the effect could differ, making extrapolation to *in vivo* events difficult. In the case of lead, the discrepancy may be readily reconciled by understanding the context in which the *in vitro* and *in vivo* measurements were made. Acute exposure to Pb^{2+} is well known to directly inhibit the NMDA receptor channel (e.g., Alkondon *et al.*, 1990). However, in the preparation of a crude membrane suspension from chronically exposed tissue for assessment of channel function, the accumulated Pb^{2+} is washed away. Thus, the preparation reflects the effects of long-term exposure without the physical presence of the metal ion. The resulting increased density of NMDA receptors suggests a compensatory up-regulation in response to long-term inhibition of the channel (Lasley and Gilbert, 1998), due perhaps in part to the direct effect of Pb^{2+}.

Understanding of the interaction of toxicant with intracellular target can resolve differences between results *in vivo* and *in vitro*, as discussed above. However, there are times when the *in vivo/in vitro* discrepancy is not resolvable. The effect of lead on astrocytes provides an example. Rat astrocyte cultures exposed to lead demonstrated increased glutathione levels and decreased mitochondrial-membrane potentials, but astroglial cultures prepared from the cerebral cortices of rat pups exposed to lead had increases in both glutathione levels and mitochondrial membrane potentials (Tiffany-Castiglioni *et al.*, 1998).

The relationship between toxicant concentrations needed to cause effects *in vitro* and those relevant to *in vivo* toxicities continues to be an area of contention among toxicologists. Although relevance increases as concentrations used *in vitro* approach those that occur in the body when the same effects are observed, concentration differences might occur for a number of reasons. Therefore, it would be unwise, within certain limits, to disregard data because they were obtained at higher concentrations than those expected in animals. For example, as mentioned above, chronic Pb^{2+} exposure in rats produced effects on long term potentiation that were qualitatively similar, but dissimilar in terms of dose-dependent responses between *in vivo* and *ex vivo* preparations (Lasley and Gilbert, 1998). In order to reconcile the dose differences, the context in which data were collected must be considered. In particular, testing paradigms for assessing potentiation in intact animals

and in hippocampal slices are different, and slices lack some recurrent neuronal circuitry. In addition, the concentration and speciation of heavy metals such as Pb^{2+} in brain after chronic *in vivo* exposure is essentially unknown. This situation currently eliminates the possibility of comparing intracellular and extracellular concentrations of the free ion with those employed for *in vitro* studies.

Choice of the *in vitro* system may be one contributing factor to concentration differences in susceptibility to toxicants. For example, continuous cell lines provide technical and economic advantages for screening large numbers of chemicals (Veronesi, 1992). However, continuous cell lines are of neoplastic origin and, consequently, are often extremely hardy and unsusceptible to lethal effects of many toxicants, except at very high concentrations. This resistance, including species differences, can be observed with neuroblastoma cells exposed to organophosphates. For example, the millimolar concentrations of organophosphate insecticides needed to kill these cells are higher than those needed to kill animals. However, acetylcholinesterase inhibition occurs at concentrations that are relevant *in vivo* (Ehrich *et al.*, 1997; van den Beukel *et al.*, 1998; Veronesi *et al.*, 1997). Even when primary cultures or *ex vivo* systems are used, dosage regimen, animal age, capacity for compensatory mechanisms, methods of tissue preparation, and inclusion of multiple cell types or elimination of recurrent neuronal circuits could modify the nature of toxicant-induced alterations from those seen in the intact animal (Lasley and Gilbert, 1998; Tiffany-Castiglioni *et al.*, 1998). Concentration-response evaluations are especially important for *in vitro* studies, regardless of the type of system used, and time-response observations are also valuable when evaluating effects *in vitro* in relation to those seen *in vivo*.

New *in vitro* reductionist systems are currently being developed, including those that contain mutant gene products, which have great potential for mechanistic studies of neurodegenerative diseases and neurotoxicant-induced injury. These include the *in vitro* model for amyotrophic lateral sclerosis, which uses primary murine spinal cord cultures with mutant Cu/Zn-superoxide dismutase-1 (Durham *et al.*, 1997, 1998; Roy *et al.*, 1998). Investigators should be aware, however, that the use of genetically manipulated *in vitro* systems could provide some spurious results. For example, human muscarinic receptors, when inserted into Chinese hamster ovary cells, did not show the same sensitivity to the test organophosphates used as did the muscarinic acetylcholine receptors in the human brain itself (van den Beukel *et al.*, 1998).

Appropriate controls are essential when performing *in vitro* experiments. Days in culture and developmental stage of the system may cause experimental variability. These protocols should be reported and synchronized, in order to enhance the reproducibility of experimental results in other laboratories, as alternative methods undergo validation and general scientific acceptance. The timing of exposure to neurotoxicants, in relation to the developmental stage of the system, should also be specified as results are reported. As more and more neurotoxicant investigations are carried out with *in vitro* systems, it becomes increasingly important that the investigators know the systems being used and their context, including factors that contribute to variability and phenotypic limitations, especially when attempts are made to extrapolate to events that occur in whole animals exposed to neurotoxicants. When used properly, however, *in vitro* studies of neurotoxicity provide advantages both for mechanistic studies and for screening of neurotoxic chemicals (Abdulla and Campbell, 1997; Campbell *et al.*, 1996; Halks-Miller *et al.*, 1991; Nelson and Brenneman, 1994; Veronesi, 1992). The discussion above suggests that it is possible to "see the world in a grain of sand" as long as controls, intracellular targets, and dose-response are considered.

Tinkering with Peter Pan Cells

The use of continuous cell lines requires special consideration in this discussion of neurotoxicity testing, because it is viewed with undeserved levels of both acceptance and disapproval. Unlike cells in the animal body, most continuous cell lines do not undergo predictable and reliable stages of development, maturation, and aging, and like Peter Pan, they may stay forever young. Whereas the developmental stage of the brain at the time of its exposure to a neurotoxicant is often predictive of the severity and type of damage incurred, *in vitro* models must attempt to mimic appropriate developmental stages or ages. Cell cultures, particularly cell lines, provide limited precision in control of the developmental stage. Some cell lines are famous for reliably getting old in culture, such as WI-38 human fibroblast cells, which undergo senescence and serve as a model for aging. Other cell lines are equally famous for being immortal, and never growing aged enough to undergo senescence. Tumor-derived and transformed cells, which include most neuronal and glial cell lines, fall into the latter category. How can one use "Peter Pan cells" that never grow up to obtain valid toxicologic data?

The principal approach is to limit the scope of questions asked so that they are an appropriate phenotype for the cell-culture model. Thus, if the cells express properties that are "normal" or relevant to the proposed mechanism of action of a neurotoxicant, they may be useful models. However, this experimental approach must be used with good judgement, not merely to take advantage of the fact that Peter Pan cells are easy to culture and obtain in large numbers. A transformed or tumor-derived cell has a split personality. It can be a cell that remains youthful, but contains chromosomes that are corrupt. Several cell lines used by the authors for neurotoxicity studies are described in this section. Typically, these *in vitro* studies have focused on the immature nervous system. This is done in part because it is more vulnerable than the mature nervous system to neurotoxicants such as lead and ethanol, and in part because the mature or aging nervous systems are more difficult to represent *in vitro*.

Examples of continuous cells used for toxicity studies are the C6 rat glioma cell line, the 1321N1 human astrocytoma cell line, and the human neuroblastoma SH-SY5Y cell line. Late-passage C6 cells, which are primarily astrocytic (reviewed by Vernadakis and Kentroti, 1996), have been used to identify two distinct mechanisms for intracellular retention and accumulation of lead: blockage of ion channels that are not Ca^{2+} channels of the L-type, and complexation of internal sulfhydryl groups (Qian *et al.*, 1999; Tiffany-Castiglioni *et al.*, 1988). The human astrocytoma 1321N1 cell line has been used to study how glial proliferation, stimulated by the activation of muscarinic receptors, is inhibited by ethanol. In this case, the developmental immaturity of the cell line mimics the appropriate target cells *in vivo*: rapidly dividing glia during the brain growth spurt. The effects of ethanol on proliferation are similar in astrocytoma 1321N1 cells and in primary cultures of astrocytes (Guizzetti *et al.*, 1996). The SH-SY5Y human neuroblastoma cell line has a stable, nearly diploid karotype and a neuronal phenotype, making it useful for studies of neuronal differentiation, growth inhibition, and neurotransmitter release and reception, as well as for studies of lead and organophosphate neurotoxicity (Ehrich *et al.*, 1997; Ehrich and Correll, 1998; Perez-Polo *et al.*, 1979; Reuveny and Narahasi, 1991; Tiffany-Castiglioni *et al.*, 1986). The stage of differentiation of most of these cell lines can be manipulated by growth factors, which influence maturity or phenotype. Thus, nerve growth factor enhances the adrenergic neuronal phenotype of SH-SY5Y cells (Perez-Polo *et al.*, 1979).

If continuous cell lines present difficulties in culture because they do not age, does the use of primary cultures circumvent this difficulty? Primary cultures eventually cease to divide, but the accuracy with which they age is problematic. Astroglia, ependymal cells, and oligodendroglia, in dissociated cultures from embryonic rat brain, appear to age in culture because they express cell-specific markers "on schedule," suggesting that their biological clocks are operational at least in early development (Abney *et al.*, 1981). The elapse of time in culture may not be sufficient, however, to guarantee appropriate differentiation, particularly in cell cultures homogeneous for one cell type. For example, Tiffany-Castiglioni *et al.* (1987) once took the view that astroglial cultures of different ages could be used to assess the age-dependent vulnerability of astroglia to lead exposure. The reasoning was that, in using *in vivo* cells under high-exposure conditions, mature but not immature astroglia sequester lead in extra-mitochondrial sites and hypothetically reduce or prevent neuronal exposure (Holtzman *et al.*, 1984). They found that younger cultures of astroglia accumulated more lead intracellularly than did older astroglia, an age difference that seemed in agreement with the greater vulnerability of the immature than the mature brain to lead neurotoxicity. However, later data indicated that this is an oversimplified model because it shows that older astroglia exposed to conditioned medium from SH-SY5Y cells or co-cultured with SH-SY5Y cells can more than double their lead accumulation (Tiffany-Castiglioni *et al.*, 1998). These observations indicate that maturation and age are not the same thing after all in astroglial cultures.

Primary neuronal cultures, too, change with time after they are isolated from various tissues. The proportion of various cell types and fiber outgrowth are among the changes that can occur (Banker and Goslin, 1991). One preparation that avoids the former problem is granule-neuron primary cultures from the rat cerebellum. These cultures have been used by a number of investigators because they are well characterized for studying receptor-mediated mechanisms. The purity of these cell preparation is >95%, the cells are more homogeneous than other primary neurons, and the target for toxicant attack might be cerebellum. A large body of information is also available with this cell preparation in terms of understanding toxicant-induced Ca^{2+} disposition and the neurotoxicity of methylmercury (Marty and Atchison, 1998; Sarafian, 1993) and PCBs (Tilson and Kodavanti, 1997, 1998).

The dissociated spinal cord cultures discussed in this paper were used in experiments 4 to 7 weeks following dissociation (Durham *et al.*, 1998). During this time, *in vitro* motor neurons differentiate to resemble their counterparts in intact spinal cord, both morphologically and by expression of biological markers and in their response to neurotoxicants (Roy *et al.*, 1998). The most obvious deficiency noted has been the lack of axonal myelination, although Schwann cell contact is established.

Although both continuous cell lines and primary cultures have advantages and disadvantages, both have a place in neurotoxicological studies. The investigator, however, needs to know and understand the system being used.

Fishing for Bait to Catch the Marlin

Throughout the brief history of our discipline, neurotoxicologists have relied heavily on advances in basic neuroscience for both scientific theoretical and methodologic advances, and thus much modern molecular and cellular toxicologic research is carried out by applying toxicants to experimental preparations that were developed to address fundamental questions in neuroscience. This situation is changing. Innovative approaches in neurotoxicology are beginning to contribute to an improved understanding of normal nervous system physiology. Therefore, both neurotoxicologists and neuroscientists are providing the "bait to catch the marlin," or the basic information to examine mechanisms of toxicity.This development is particularly well illustrated by the synergetic use of *in vitro* and *in vivo* methodologies to examine neurotoxicity of PCBs, as well as the effects of alcohol on the developing female neuroendocrine system.

Usage of *in vitro* preparations such as primary-granule neurons and isolated subcellular organelles has contributed to a preliminary understanding of neurotoxic effects of PCBs. It supports the hypothesis that some PCBs may act directly in the

central nervous system. *In vitro* results indicate that only *ortho* PCB congeners that do not bind or weakly bind to aryl hydrocarbon (Ah) receptors affect signal transduction/second messenger systems (Kodavanti and Tilson, 1997; Tilson and Kodavanti, 1997) that play key roles in neuronal development and function. These results challenge the prevailing model that all PCB effects are mediated by the Ah receptor. *In vitro* findings parallel several *in vivo* results, including PCB-induced neurochemical changes and alterations of second messenger systems (Fischer *et al.*, 1998; Kodavanti, 1998).

Parallel *in vitro/in vivo* experimental approaches have also been used creatively for hypothesis development in elucidating neuroendocrine mechanisms by which ethanol can delay female puberty. In this case, much of the endocrinology had to be worked out first. For insulin-like growth factor-1 (IGF-1) to be a metabolic signal at the time of female puberty, it must be capable of inducing the release of luteinizing, hormone-releasing hormone (LHRH) from the hypothalamus. Because IGF-1 is very expensive, preliminary evidence was obtained *in vitro*, where IGF-1-induced LHRH release was indeed found via incubation of hypothalami (Hiney *et al.*, 1991). This information led to a number of elaborate and expensive *in vivo* experiments with physiological and molecular techniques, which ultimately showed that IGF-1 of peripheral origin acts centrally to accelerate the initiation of female puberty in the rat (Hiney *et al.*, 1996). This effect has also been demonstrated in the rhesus monkey (Wilson, 1998). These assessments opened the door for examining potential detrimental effects of toxic substances, such as ethanol, on the role of IGF-1 at puberty. Once the *in vivo* studies revealed the site of action and the role for IGF-1 in the pubertal process, as well as in drug-induced alterations, it became apparent that *in vitro* methodologies could be used again, but this time for much different reasons. They could be used to determine the mechanism of the IGF-1 action and assess the effect of alcohol on that action. These *in vitro* studies revealed that IGF-1-induced LHRH release is mediated by prostaglandin E2 and this effect is blocked by alcohol. (Hiney *et al.*, 1997; Dees *et al.*, 1998).

These examples clearly show that *in vitro* and *in vivo* methods can be complementary to each other, and that this approach is useful for advancing specific areas of biomedical/toxicological research. The combined use of these methods could be likened to "goin' fishin'"; one can be used to catch the bait, and the other to catch the marlin.

CONCLUSIONS

Among the themes discussed were developmental parallels, validation of *in vitro* and *ex vivo* observations, and improvement of reductionist models. The authors of this commentary have come to a consensus that the validity of *in vitro* models depends on at least three factors: endpoints measured, dosing level and regimen, and cell interactions. Thus, as a first step towards integrating *in vitro* studies with *in vivo* toxicant effects, studies done with single cell types should begin to reflect processes that are part of the toxicant action at a higher level. Well-chosen endpoints will have potential for contributing to understanding of mechanisms of toxicant action in systems that are more complex. A second step should be to accurately estimate toxicologically relevant doses and dose regimens. These should be based on such factors as tissue levels, metabolism, bioavailability, and pharmacodynamics of the toxicant with regard to putative target cells, as well as age at time of exposure and number of exposures. A third step for improving *in vitro* toxicity testing should be to include experimental models in which heterogeneous cell interactions are preserved at the time of exposure. Cell interactions in the central and peripheral nervous systems are essential components of tissue function, but they are traditionally discarded in the interest of simplicity in neurotoxicity testing *in vitro*. These commonly recognized issues are difficult to adequately address with *in vitro* models, although considerable recent progress has been made.

It is also important to keep in mind the conditions distinguishing *in vitro*, *ex vivo*, and *in vivo* models. *In vitro* models often represent only the acute or short-term effects of the toxicant, while *ex vivo* preparations represent longer-term-treatment effects carried over into *in vitro* conditions. *In vivo* models include longer-term-treatment effects and the influences of biological integration. Thus, the improvement of *in vitro* reductionist models should include not only the construction of complex culture systems that replicate tissue functions, but also include the analysis of *ex vivo* preparations (cell cultures, organ cultures, explants, or tissue slices from exposed animals) that preserve abnormalities induced by *in vivo* neurotoxicant exposure. The ideas and examples discussed in this paper may provide an additional framework in which to consider how best to tackle the issues of neurotoxicity testing in experimental models.

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